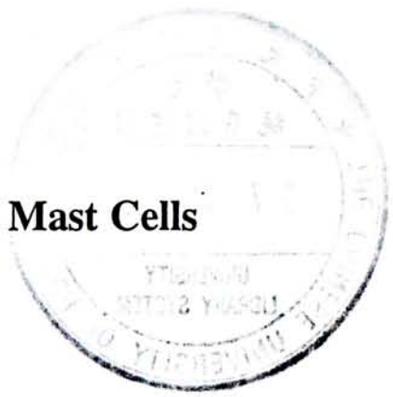
A thesis presented to The Chinese University of Hong Kong in partial fulfilment of the requirements for the degree of Doctor of Philosophy

Effects of Phospholipase A_2 on Mast Cells



by

Catherine Mary (Kay) Roche B.Sc.

Department of Pharmacology
Division of Basic Medical Sciences
The Chinese University of Hong Kong

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Abstract

In this study, the effects of the $sPLA_2$ enzymes isolated from the snake venoms of *Naja naja* venom ($sPLA_2$-I) and *Crotalus altrox* venom ($sPLA_2$-II) on mast cell reactivity were examined. Mast cells isolated from the rat peritoneal cavity were chosen as a model to examine the effects of these $sPLA_2$ enzymes.

Mast cells derived from the rat differed in their response to these $sPLA_2$ enzymes. Histamine was released in a dose dependent manner by $sPLA_2$-I up to a maximum of 68% of the total histamine content. This was dependent on extracellular Ca^{2+}, metabolic energy and the native structure of $sPLA_2$-I. This histamine release was significantly reduced by the PLA_2 inhibitors, *p*-BPB and 12-epi-scalaradial and was unaffected by the cyclo-oxygenase inhibitor, flurbiprofen and the lipoxygenase inhibitor, zileuton. In contrast, the maximum % histamine release observed with $sPLA_2$-II was generally not greater than 10%. This low level of histamine release was blocked by the PLA_2 inhibitors and was unaffected by flurbiprofen and zileuton pretreatment. In addition, PGD_2 was produced dose dependently with $sPLA_2$-II, whereas with $sPLA_2$-I production was only observed with a high concentration of 1 U ml^{-1}. It is proposed that the enzymatic nature of the $sPLA_2$ enzymes was involved in mediating the effects observed here. In addition a receptor mediated response is also possible as binding sites for these $sPLA_2$ enzymes have recently been identified. Further studies are required to establish the exact mechanism (s) involved in these cellular responses.

Rat mast cells activated by different stimuli (anti-IgE, compound 48/80 and A23187) differed in their response to the $sPLA_2$ enzymes. Immunologically induced histamine release was enhanced in the presence of the $sPLA_2$ enzymes. This enhancement was reduced by the PLA_2 inhibitors and was unaffected by flurbiprofen and zileuton. Immunologically induced PGD_2 production was enhanced with $sPLA_2$-II, but not with $sPLA_2$-I. The $sPLA_2$ enzymes did not enhance histamine release when mast cells were activated with compound 48/80 or the calcium ionophore A23187.

The mechanism of this enhancement was further investigated using intracellular

calcium measurements. Both $sPLA_2$ enzymes led to a slow increase in the $[Ca^{2+}]_i$. The net increase in the $[Ca^{2+}]_i$ was higher with $sPLA_2$-I when compared with $sPLA_2$-II. However, $sPLA_2$-II produced a significant increase in the $[Ca^{2+}]_i$ following activation with anti-rat IgE. This enhancement was specific for the late phase of the biphasic calcium response. No enhancement of the Ca^{2+} signal was observed with $sPLA_2$-I. It is proposed that the $sPLA_2$-II plays a role in the calcium influx pathway required for the immunological activation of mast cells. Further studies are required to establish the exact mechanism(s) of action of $sPLA_2$-II.

This study also demonstrated the heterogeneity of mast cells isolated from the rat peritoneum, human lung and guinea pig lung tissues, in their responsivity to the $sPLA_2$ enzymes. Histamine release was observed with mast cells from the rat and guinea pig (high concentrations of $sPLA_2$-I), whereas human lung mast cells were generally unresponsive. Mast cells isolated from all three sources were generally unresponsive to $sPLA_2$-II (the maximum % histamine release was about 10%). Immunologically induced histamine release from rat mast cells was enhanced in the presence of these $sPLA_2$ enzymes. In contrast, inhibition was observed with mast cells isolated from human and guinea pig lung tissues.

The present study indicates two potential roles for $sPLA_2$ enzymes in immunological reactions. Studies with rat peritoneal mast cells have suggested a pro-inflammatory role whereas the human studies would suggest an anti-inflammatory role. This highlights the importance of studying precise mast cell populations in relation to inflammatory conditions and in the development of agents to inhibit the effects of these $sPLA_2$ enzymes.

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Publications based on work in this thesis

Papers

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LAU, H.Y.A. & ROCHE, C.M. (1995). Effects of secretory PLA_2 on rat peritoneal mast cells activated by different secretagogues. In *Eicosanoids & Other Bioactive Lipids in Cancer, Inflammation & Radiation Injury*. eds. Honn, K.Y., Nigam, S., Jones, R., Marnette, L.J. and Wong, P.Y-K. In press.

Abstracts

ROCHE, C.M., LAU, H.Y.A. (1994). Effects of type I and type II phospholipase A_2 on rat peritoneal mast cells. *Proceedings of the 23rd meeting of the European Histamine Research Society.*

LAU, H.Y.A. & ROCHE, C.M. (1994). Effects of secretory phospholipase A_2 on mast cells. *Can. J. Physiol, Pharmacol.*, **72**, (Suppl., 1), 279.

ROCHE, C.M. & LAU, H.Y.A. (1995). Effects of secretory phospholipase A_2 on rat peritoneal mast cells activated by different secretagogues. *Proceedings of the 4th International Conference on Eicosanoids & Other Bioactive Lipids in Cancer, Inflammation & Radiation Injury*. 54.

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Abbreviations

12-epi-scalaradial	12-deacetyl-12-epi-scalaradial
AA	Arachidonic acid
Anti-human IgE	Goat anti-human IgE serum
Anti-rat IgE	Sheep anti-rat IgE serum
ATP	Adenosine triphosphate
BMMC	Mouse bone marrow-derived IL-3-dependent mast cells
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CMC	Critical micelle concentration
Con A	Concanavalin A
$cPLA_2$	Cytosolic PLA_2
CTMC	Connective tissue mast cell
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
FCS	Foetal calf serum
G-protein	Guanine nucleotide binding protein
GPLMC	Guinea pig lung mast cells
GppNHp	Guanosine 5'-(β,γ-imido)triphosphate
GTP-β-S	Guanosine 5'-O-(2-thiodiphosphate)
GTP-γ-S	Guanosine 5'-O-(3-thiotriphosphate)
HLMC	Human lung mast cells
I_{CRAC}	Calcium release-activated calcium current
IgE	Immunoglobulin E
IL	Interleukin
IP_3	Inositol 1,4,5-trisphosphate
$iPLA_2$	Calcium independent phospholipase A_2
K_d	Equilibrium binding constant
LPS	Lipopolysaccharide
LysoPC	Lysophosphatidylcholine

LysoPE	Lysophosphatidylethanolamine
LysoPS	Lysophosphatidylserine
MAFP	Methyl arachidonyl fluorophosphonate
MMC	Mucosal mast cell
OVA	Chicken egg ovalbumin/ovalbumin
p-BPB	*p*-Bromophenacyl bromide
PAF	Platelet-activating factor
PBS	Phosphate buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PGD_2	Prostaglandin D_2
PGD_2-MOX	Prostaglandin D_2 methoxime
PI	Phosphatidylinositol
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PLD	Phospholipase D
PS	Phosphatidylserine
PTK	Protein tyrosine kinase
RBL-2H3	Rat basophilic leukemic cells
RMCP	Rat mast cell protease
RPMC	Rat peritoneal mast cells
SCF	Stem cell factor
SEM	Standard error of the mean
$sPLA_2$	Secretory phospholipase A_2
$sPLA_2$-I	Type I $sPLA_2$
$sPLA_2$-II	Type II $sPLA_2$
TNF	Tumor necrosis factor

CONTENTS

CHAPTER 1

INTRODUCTION

1.1 Historical background / General information

Mast cells were originally defined by Ehrlich as mononuclear cells containing metachromatic granules, due to the presence of proteoglycans such as heparin. The granules of these cells took up blue dyes and changed colour to a pinkish/purple colour. They were first seen in unstained frog mesentery preparations by Von Recklinghausen in the latter half of the nineteenth century. Ehrlich named the cell "Mastzelle" from the German word "mästen", which means to fatten or feed, as he thought these cells represented over-nourished connective tissue cells. He also described the mast cell's circulating blood counterpart, the basophil. Mast cells and basophils share two basic properties: secretory granules and high affinity receptors for immunoglobulin E (IgE) (for review see Bloom, 1984; Wasserman, 1990; Marone, 1995). It was recognised in 1953, that these cells contained histamine in their secretory granules (Riley & West, 1953). At the same time, histamine was shown to be one of the mediators of acute allergic reactions.

It is now clear that the mast cell plays a major role in mediating the symptoms of immediate hypersensitivity reactions (White & Kaliner, 1988; Dale & Foreman, 1989; White, 1990; Pearce, 1991; Longlet *et al.*, 1995). The mast cell is uniquely placed to participate in allergic responses and populates tissues which come into frequent contact with the external environment such as the skin, the respiratory and gastrointestinal tract (Lichtenstein, 1993). More recently mast cells have also been found associated with the brain (for review see Silver *et al.*, 1996).

Mast cells are now known to exist as many different types and display marked heterogeneity in their morphological, histochemical and functional properties (for review see Barrett & Pearce, 1993; Irani & Schwartz, 1994; see chapter 4). Morphologically mature mast cells are large (9 to 13 μm), have a single nucleus and a cytoplasm packed with large membrane bound granules. Human mast cells also contain numerous cytoplasmic lipid bodies (Behrendt *et al.*, 1978; Dvorak *et al.*, 1983; Galli *et al.*, 1984).

Mast cells are thought to originate from multipotential stem cells in the bone marrow (for review see Kitamura *et al.*, 1993a & b). These undifferentiated mast cell precursors leave the bone marrow, migrate in the blood and invade connective and mucosal tissues where differentiation and proliferation is completed. Mast cell development is regulated by T cell and fibroblast derived growth factors, interleukin-3 (IL-3) together with stem cell factor (SCF) possibly being the most important (Galli *et al.*, 1993; Kitamura *et al.*, 1993a & b).

1.2 Mast cell mediators

The activation of mast cells causes the release of a wide range of biologically active compounds. These mediators are either preformed and stored within the secretory granule, or are rapidly synthesised following cell activation. The former includes histamine, proteoglycan and proteinases. The latter includes arachidonic acid (AA) metabolites such as the prostaglandins (PG) and leukotrienes (LT), platelet-activating factor (PAF), and a variety of cytokines (for review see Harvima & Schwartz, 1993).

1.2.1 Pre-formed mediators

Histamine, the dominant amine stored in mast cells and basophils, is synthesised from histidine by L-histidine decarboxylase (Bauza & Lagunoff, 1981). It is stored in the cytoplasmic secretory granules (acidic pH), where it is positively charged and appears to bind to negatively charged groups in the proteoglycan-protein matrix. Upon cellular activation, histamine rapidly dissociates from the proteoglycan-protein matrix by cation exchange with extracellular sodium at neutral pH (Uvnäs, 1970). Histamine released following cellular degranulation rapidly diffuses into the surrounding tissues where it exerts its biological effects by binding to H_1, H_2 and H_3 receptors on cell surfaces (White & Kaliner, 1988; White,1990; Leurs *et al.*, 1995). The effects mediated via the H_1 receptor include bronchial and gastro-intestinal smooth muscle contraction and increased permeability of the venular endothelium. In contrast, the effects mediated via the H_2 receptor are more anti-inflammatory and include the suppression of T-lymphocyte cytotoxicity, suppression of lymphocyte proliferation, stimulation of gastric acid secretion and enhanced epithelial permeability in human airways. H_3 receptors regulate histamine formation and release in the brain. Rat peritoneal mast cells have high histamine levels of 10-30 pg/cell whereas human mast cells, basophils and rat mucosal mast cells have low levels of 1-3 pg/cell (Harvima & Schwartz, 1993). Connective tissue mast cells of the rat, but not of man, also contain the amine serotonin (5-hydroxytryptamine) (Weitzman *et al.*, 1985).

The presence of highly sulphated negatively charged proteoglycans in the secretory mast cell granules remains the principal method of visualizing mast cells.

Histochemical staining of these cells with basic dyes results in metachromasia as seen under the light microscope. Two major sub-classes of proteoglycans have been identified in mast-cells: heparin and chondroitin sulphate. Heparin is the principal proteoglycan of human mast cells, as well as rat and mouse connective tissue mast cells. Rat mucosal mast cells contain chondroitin sulphate. These mast cell proteoglycans bind to histamine, neutral proteases and acyl hydrolases at the acidic pH within the secretory granules and are thought to facilitate the uptake and packaging of preformed mediators into the secretory granules, in addition to regulating their stability and enzymatic activity (Harvima & Schwartz, 1993).

Mast cell granules also contain a number of enzymes such as exoglycosidases, arysulphatases and neutral proteases. The former are found loosely bound to the structural matrices and are released in parallel with histamine upon cell activation. Neutral proteases catalyse peptide bond cleavage and comprise about a third of the total protein of mast cell granules and serve as markers for distinguishing different cell types (Harvima & Schwartz, 1993).

1.2.2 Newly synthesised lipid mediators

Some mediators are only formed on activation of the mast cell. Endogenous AA is the most important precursor of these mediators (Sullivan & Parker, 1979). This unsaturated fatty acid is released from the membrane glycerolphospholipids by the action of the enzyme phospholipase A_2 (PLA_2), or phospholipase C (PLC) together with diacylglycerol lipase and/or other lipases. Once liberated, AA is oxidatively

metabolised by several pathways to produce thromboxanes, prostaglandins and leukotrienes collectively known as the eicosanoids. The cyclo-oxygenase pathway, which gives rise to prostaglandins and thromboxanes, starts with the action of prostaglandin endoperoxide synthase (Smith, 1989). In both human and rat mast cells the major product released is prostaglandin D_2 (PGD_2) (Lewis *et al.*, 1982; Ennis *et al.*, 1984). PGD_2 is a short lived constrictor of bronchial smooth muscle (Hardy *et al.*, 1984) and is a potent inhibitor of platelet aggregation (Mills & MacFarlane, 1974). The lipoxygenase pathway first generates the unstable compound 5-hydroperoxyeicosatetraeonic acid (5-HPETE), which is rapidly metabolised to 5-hydroxyeicosatetraenoic acid (5-HETE) and LTA_4. LTA_4 can be metabolised to yield LTB_4 or LTC_4(Smith, 1989). LTC_4 and LTB_4 are generated by human lung mast cells (Peters *et al.*, 1982b; Freeland *et al.*, 1988). LTB_4 is a potent chemotaxin for neutrophils and eosinophils (Ford-Hutchinson *et al.*, 1980; Goetzl & Pickett, 1981). The mixture of leukotrienes LTC_4, LTD_4 and LTE_4 have many physiological effects such as enhanced vascular permeability, constriction of bronchial smooth muscle and enhanced mucus secretion (Lewis *et al.*, 1990). In rodents LTC_4 and LTB_4 are generated by mucosal mast cells rather than by connective tissue type mast cells (Schwartz, 1987).

Platelet-activating factor (PAF) is another lipid mediator generated during inflammatory reactions. Activated human lung mast cells generate PAF whereas basophils do not. PAF activates neutrophils to release mediators involved in chemotaxis, causes smooth muscle contraction, enhances vasopermeability and is chemotactic for eosinophils (Schwartz, 1987).

In addition, mast cells can synthesise and release a variety of functional cytokines such as IL-3, IL-4, IL-5, IL-6, granulocyte-macrophage colony stimulating factor (GM-CSF), tumour necrosis factor (TNF)-α and interferon (IFN)-γ (Galli *et al.*, 1991; Galli, 1993; Marshall & Bienenstock, 1994; Galli & Costa, 1995). Cytokine synthesis occurs hours after activation in contrast with the rapid generation of AA metabolites. The functions of these cytokines have not yet been established, but are thought to be involved in mast cell differentiation.

1.3 Mast Cell Activation

Two pathways of mast cell activation have been extensively characterised: the antigenic pathway and the non-antigenic pathway (for review see Landry *et al.*, 1992; Sagi-Eisenberg 1993; Foreman, 1993). Following activation, mediators such as histamine and PGD_2 are released into the extracellular environment.

1.3.1 The antigenic pathway of mast cell activation

The classical pathway of activation involves IgE, produced by B lymphocytes. Contact of a foreign allergen with a susceptible individual, stimulates B-cell differentiation into IgE antibody secreting cells and a population of B-memory cells. The allergen also interacts with T-lymphocytes which augment or inhibit IgE production via T-helper or T-suppresser cells respectively (Vercelli & Geha, 1989).

The IgE molecules produced bind to high affinity IgE receptors ($Fc_\varepsilon R1$) found on mast

cell membranes (Ishizaka & Ishizaka, 1974). Early classical studies demonstrated that bridging of specific receptors of the IgE type by the corresponding allergen triggered mediator release. Other agents are also able to cross-link IgE receptors such as dimerised IgE, anti-IgE and con A (a lectin which cross-links IgE carbohydrate moieties) and can cause mediator release (Keller, 1973; Langunoff & Martin, 1983; Foreman, 1993; Razin *et al.*, 1995).

Molecular characterisation of the high affinity IgE receptor revealed a tetrameric complex made up of an α and β sub-unit, and two γ sub-units linked by disulphide bonds. The extracellular domain of the α sub-unit is necessary for IgE binding. The carboxyl-terminal cytoplasmic tails of both the β and γ sub-units, buried in the cell membrane, are important in Fc_eR1 mediated signal transduction (Metzger, 1992; Alber & Metzger, 1993). These sub-units do not display any enzymatic activity by themselves but are quickly and transiently phosphorylated following receptor crosslinkage (for review see Hamawy *et al.*, 1995).

Aggregation of the high affinity IgE receptor molecules on the mast cell membrane, with allergen, initiates an intracellular signalling pathway, that results in mast cell degranulation and the release of inflammatory mediators. The exact sequence of events which occurs following receptor aggregation and subsequent mediator release has yet to be determined. At present many enzyme systems and cell components are thought to be involved (for reviews see Sagi-Eisenberg, 1993; Beaven & Metzger, 1993; Razin *et al.*, 1995; Scharenberg & Kinet, 1995).

The phosphatidylinositol system appears to be one of the major pathways activated upon IgE receptor aggregation. Hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLC in the cell membrane, leads to the concomitant release of diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 itself acts as an intracellular second messenger and releases calcium from intracellular stores, resulting in an increase in the intracellular calcium ion concentration ($[Ca^{2+}]_i$), which is usually transient (for review see Berridge, 1993).

An increase in the $[Ca^{2+}]_i$ is required for histamine secretion. The direct microinjection of Ca^{2+} into mast cells (Kanno *et al.*, 1973), together with the use of Ca^{2+} ionophores (Foreman *et al.*, 1973) both provide evidence for the key role of Ca^{2+} in mast cell degranulation. Liposomes containing calcium, but not magnesium or potassium ions were also shown to induce histamine release from rat peritoneal mast cells (Theoharides & Douglas, 1978).

In order to maintain an elevated $[Ca^{2+}]_i$, a Ca^{2+} influx from the extracellular environment is required. Foreman *et al.* (1977) showed that IgE-mediated histamine release from rat peritoneal mast cells was accompanied by an influx of $^{45}Ca^{2+}$ from the external medium. White *et al.* (1984) used the fluorescent probe quin-2 to directly demonstrate a rise in the cytosolic $[Ca^{2+}]$ in mast cell IgE induced histamine release.

Recent patch clamp studies and single cell Ca^{2+} measurements have demonstrated the existence of two calcium influx pathways in mast cells; a calcium specific pathway activated by the depletion of calcium from intracellular stores (Hoth & Penner, 1992

& 1993; Fasolato *et al.*, 1994) and nonspecific cation channels which are permeable to divalent cations (Penner *et al.*, 1988; Matthews *et al.*, 1989; Kudo & Kimura, 1992; Fasolato *et al.*, 1993a; Obukhov *et al.*, 1995).

The calcium specific pathway was termed I_{CRAC} (for calcium release-activated calcium current). I_{CRAC} is highly selective for Ca^{2+}, over Ba^{2+}, Sr^{2+} and Mn^{2+}. I_{CRAC} is activated following depletion of the intracellular calcium stores, either by IP_3, ionomycin or EGTA. It is voltage independent and inhibited by high cytosolic $[Ca^{2+}]$ (Hoth & Penner, 1992 & 1993; Fasolato *et al.*, 1994). The capacitance model described by Putney (1990) links the depletion of internal calcium stores with the activation of a calcium influx pathway. Depletion of the intracellular calcium stores are believed to release a signal which activates this calcium influx. Randriamampita & Tsien (1993), and Parekh *et al.* (1993) have isolated a molecule thought to be involved in the activation of this calcium influx. Fasolato *et al.* (1993b) reported that the signal for this calcium influx involved a GTP-dependent step. More recently, Parekh & Penner (1995) reported that I_{CRAC} in the rat basophilic leukemia cell line (RBL-2H3) can be inactivated following treatment with the protein kinase C (PKC) activator, phorbol 12-myristate 13-acetate. The PKC inhibitors staurosporine and bisindolylmaleimide prevented inactivation of I_{CRAC} suggesting a role for PKC in its regulation. Zhang & McCloskey (1995) have reported activation of I_{CRAC} in RBL-2H3 cells following IgE receptor crosslinkage.

The nonspecific cation channels are known as the large conductance 50-pS channels (Penner *et al.*, 1988; Matthews *et al.*, 1989; Kudo & Kimura, 1992; Fasolato *et al.*,

1993a). The 50pS channel is thought to be regulated by a guanine nucleotide binding protein (G protein), as it was activated by internal guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) and inhibited by internal guanosine 5'-O-(2-thiodiphosphate) (GTP-β-S). The activity of this channel is inhibited by elevated $[Ca^{2+}]_i$. More recently, Obukhov *et al.* (1995) have reported on a large conductance (250 pS) nonselective cation channel in RBL-2H3 cells. This channel was activated by ATP and carbachol and activation was independent of internal calcium.

The efflux of calcium is thought to be mediated by a sodium calcium antiporter system. The immunological activation of mast cells causes a temporary cessation of this efflux pathway and enhances the $[Ca^{2+}]_i$ produced by the release of calcium from internal stores and the influx from the extracellular medium (Pearce & White, 1984).

The second product of phosphatidyl inositol breakdown catalysed by PLC is diacylglycerol (DAG). DAG is the endogenous activator of protein kinase C (PKC). Although protein phosphorylation is the role of this calcium dependent enzyme, its role in mast cell activation is yet to be determined (Chakravarty, 1990; White *et al.*, 1990b). Recent studies, have suggested that a phosphatidylcholine (PC) cycle may be a better source of DAG, involving phospholipase D (PLD) (Kennerly, 1990; Exon, 1994).

IgE receptor cross linking appears to initiate a series of protein phosphorylation reactions (for review see Hamawy *et al.*, 1995). Phosphorylation of the β and γ sub-units, by non-receptor tyrosine kinases is rapid and requires aggregated receptors. The

src-family protein-tyrosine kinase (PTK) $p53/56^{lyn}$ (Eiseman & Bolen, 1992; Field *et al.*, 1995) and the PTK $p72^{syk}$ (Takanobu *et al.*, 1991; Benhamou *et al.*, 1993) are thought to participate in these reactions. In addition the PLC isoenzyme PLCγ1(Park *et al.*, 1991; Li *et al.*, 1992) and the δ isoform of PKC (Germano *et al.*, 1994; Haleem-Smith *et al.*, 1995) are also rapidly tyrosine phosphorylated following receptor aggregation.

The involvement of G proteins in mast cell degranulation was demonstrated by Gomperts (1983). The introduction of the nonhydrolysable analogues of GTP, GTP-γ-S or GppNHp, into the cytosol of ATP^{4-} permeabilized mast cells resulted in their degranulation in the presence of external calcium. Aridor *et al.* (1990) reported that GTP-γ-S also resulted in DAG formation and was inhibited by neomycin (an aminoglycoside antibiotic known to inhibit PI breakdown). GTP-γ-S had a biphasic effect and at high concentrations ($>$ 100 μM) inhibited DAG formation. However, Saito *et al.* (1987) observed that pretreatment of rat peritoneal mast cells with islet-activating protein pertussis toxin (IAP), had no significant effect on mediator release induced by anti-IgE. Ali *et al.* (1989) also observed that the introduction GTP-γ-S resulted in PIP_2 breakdown in RBL-2H3 cells. Wilson *et al.* (1989) reported that mycophenoloic acid an inhibitor of inosine monophosphate dehydrogenase inhibited IgE mediated histamine secretion and calcium influx in RBL-2H3 cells, whereas cholera toxin enhanced secretion (Knoop & Thomas, 1984; McCloskey *et al.*, 1988). Based on these observations two mechanisms were proposed to be involved in PLC activation in rat mast cells and RBL-2H3 cells. One mechanism involves tyrosine kinase activation and subsequent phosphorylation of PLC. The second pathway

involves G-proteins as coupling entities intervening between the IgE receptor and the PLC enzyme.

IgE receptor crosslinkage also appears to initiate phospholipid methylation. Two methyltransferases are involved in converting phosphatidylethanolamine (PE) to PC. PC is then further metabolised by PLA_2 to yield lysophosphatidylcholine (LysoPC) and free AA (Ishizaka *et al.*, 1980; Ishizaka *et al.*, 1983; Axlerod & Hirata, 1982). However, several workers have been unable to confirm these reports of an increase in phospholipid methylation, following activation of mast cells or basophils (Moore *et al.*, 1984; Holgate *et al.*, 1985; Benyon *et al.*, 1988). The origin of this discrepancy is unclear but the involvement of PE methylation in histamine secretion is questionable.

It has also been suggested that a transient monophasic rise in cyclic adenosine monophosphate (cAMP) may play an important role in mast cell degranulation. The immunological activation of mast cells produces a transient increase in the intracellular level of cAMP. Bridging of the IgE receptor molecules on the mast cell membrane are thought to activate adenylate cyclase through a GTP dependent regulatory protein. This rise in cAMP also corresponds with an increase in phospholipid methylation. However, the relationship between these two events is unclear. It has been proposed that lipid methylation may be required for the activation of adenylate cyclase or it may enhance the coupling of the IgE receptor to adenylate cyclase through an increase in membrane fluidity (for review see Pearce, 1987). The role of cAMP in histamine secretion is also controversial as it has been reported both to inhibit and induce histamine release (Holgate *et al.*, 1980).

PLA_2 activation has also been reported to play a role in the release of histamine from activated mast cells. Early studies demonstrated that exogenous AA potentiated immunologically induced histamine release from rat peritoneal mast cells and human basophils (Sullivan & Parker, 1979; Marone *et al.*, 1979). This potentiation was not seen with other fatty acids such as linoleic acid and linolenic acid. Purified PLA_2 isolated from porcine pancreas was shown to cause mast cell degranulation and this was blocked by the PLA_2 inhibitor *p*-Bromophenacyl bromide (*p*-BPB) suggesting that PLA_2 was involved in the generation of this free AA in mast cells (Chi *et al.*, 1982). Eicosa-5,8,11,14-tetraynoic acid (ETYA), a cyclo-oxygenase and lipoxygenase inhibitor, inhibited histamine release from rat mast cells and human basophils (Sullivan & Parker, 1979; Marone *et al.*, 1979). Non-steroidal anti-inflammatory drugs, which block the cyclo-oxygenase pathway of AA metabolism yielded controversial results. Histamine release was unaffected by aspirin or indomethacin (Sullivan & Parker, 1979) and enhanced by indomethacin, meclofenamic acid and aspirin (Marone *et al.*, 1979). Inhibitors of the lipoxygenase pathway 5,8,11,14-henicosatetraynoic acid (HTYA), 4,7,10,13-icosatetraynoic acid (ITYA) and 5,8,11-eicosatriynoic acid (ETYA), blocked immunologically induced histamine secretion from rat mast cells and human basophils (Marone *et al.*, 1981; Nemeth & Douglas, 1982). Magro (1982) tested a wide variety of AA inhibitors on their ability to inhibit immunologically induced histamine release from human basophils. Agents specific for the lipoxygenase pathway were effective in blocking histamine release, whereas agents inhibitory to cyclo-oxygenase, isomerases and thromboxane synthetases were ineffective. However, Morita *et al.* (1985) reported that the 5-lipoxygenase inhibitor AA-861 did not inhibit immunologically induced histamine release from human basophils. More recently,

Kuno *et al.* (1993) reported that *p*-BPB and the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA) inhibited compound 48/80 induced histamine release from rat mast cells; indomethacin was without effect. These observations led to the proposal that one or more products of the lipoxygenase pathway played a role in histamine release from mast cells. Three candidates have been proposed: 12-hydroxyeicosatetraenoic acid, 5-HETE and 5-HPETE. All these compounds have been reported to significantly enhance immunologically induced histamine release from rat mast cells and human basophils (Stenson *et al.*, 1980; Peters *et al.*, 1981 & 1982a).

1.3.2 The non-antigenic pathway of mast cell activation

This pathway of mast cell activation can be selective or cytotoxic. The cytotoxic group of agents (e.g. the detergent Triton X 100) act by disrupting the plasma membrane, causing irreversible damage and expulsion of the intracellular contents. The non-cytotoxic compounds include polybasic agents (compound 48/80), neuropeptides (substance P), peptide hormones (bradykinin), venom peptides (mastoparan), anaphylatoxins (C3a, C5a) and calcium ionophores (A23187, ionomycin). Although little is known about their receptors or secretory mechanisms, several observations suggest that these stimuli utilise a secretory pathway different from IgE dependent stimuli (for review see Landry *et al.*, 1992; Langunoff & Martin 1983: Foreman, 1993).

The first known intracellular event of this pathway is the activation of pertussis toxin-sensitive G-proteins distal to PLC (Saito *et al.*, 1987; Aridor *et al.*, 1990; Aridor &

Sagi-Eisenberg, 1990). PLC activation results in the generation of IP_3 and DAG. PLA_2 is also activated (for review see Landry *et al.*, 1992; Foreman, 1993).

G-proteins are also involved in the non-immunological pathway of secretion in mast cells. Pretreatment of rat peritoneal mast cells with islet-activating protein pertussis toxin (IAP), results in a marked inhibition of receptor mediated histamine secretion when challenged with the polyamine compound 48/80 (Nakamura & Ui, 1984 & 1985; Saito *et al.*, 1987; Bronner *et al.*, 1990). Penner *et al.* (1987) and Aridor *et al.* (1990) observed that depletion of intracellular GTP levels inhibited compound 48/80 induced histamine release. The venom peptide mastoparan directly activates purified G-proteins, as do substance P and compound 48/80 (Higashijima *et al.*, 1990; Mousli *et al.*, 1990 a, b & c; Tomita *et al.*, 1991). Further work with bradykinin and anaphylatoxin C3a extended this hypothesis of G-protein activation via a receptor independent mechanism (Bueb *et al.*, 1990). The compounds described above are thought to induce histamine release through a direct interaction with a G-protein. However, these agents cannot penetrate the surface of the plasma membrane and reach the G-proteins. It appears that the hydrophobic portion of these compounds are involved in membrane insertion, allowing the basic portion to interact and activate the G-protein. Their ability to rapidly induce mediator release indicates the importance of cell surface events in initiating the response.

A role for sialic acid residues (negatively charged endings of membrane glycolipids and glycoproteins) of the cell surface has been proposed. Removal of these residues by neuraminidase treatment inhibits the response to peptides without altering IgE

dependent stimulation (Landry *et al.*, 1992). The authors proposed that binding of the secretory stimulus to sialic residues would facilitate the passage of the agents into the plasma membrane.

Although, the exact biochemistry between membrane signalling and granule release is still unclear, the final stage is the fusion of the membrane surrounding the granule with the membrane surrounding the whole cell, which results in histamine secretion (Lindau & Gomperts, 1991).

1.4 Phospholipase enzymes

Phospholipases are a group of enzymes that hydrolyse phospholipids, which are abundant in biological membranes, to generate free fatty acids and lysophospholipids. Phosphoglycerides are a class of phospholipids derived from glycerol and contain a glycerol backbone, two fatty acyl chains and a phosphorylated alcohol. The hydroxyl groups located at the first (*sn*-1) and second (*sn*-2) carbon atoms of the glycerol molecule are esterified to the carboxyl groups of the fatty acid chain. Normally saturated and unsaturated fatty acids occupy these *sn*-1 and *sn*-2 positions respectively. The hydroxyl group of the third carbon atom (*sn*-3) is esterified to phosphoric acid. This phosphate group can be esterified to the hydroxyl group of serine, ethanolamine, choline, glycerol or inositol (Dennis, 1983).

Although phospholipases (PL) share the same basic function of phospholipid hydrolysis they can be broadly classified into two categories (a) the acyl hydrolyases and (b) the

phosphodiesterases. The acyl hydrolyases (PLA_1 and PLA_2) cleave the ester bonds at the *sn*-1 and *sn*-2 position of the phospholipid molecule, respectively. The phosphodiesterases cleave at the *sn*-3 position on the glycerol side (PLC) or on the polar side (PLD) of the phospholipid molecule (Dennis, 1983).

1.4.1 Phospholipase A_2

Phospholipase A_2 (PLA_2) was the first phospholipase to be identified. In the early 1900's it was observed that the incubation of phosphatidylcholine with pancreatic juice or cobra venom resulted in the formation of free fatty acids (for historical review see Wittcoff, 1951). PLA_2 enzymes were subsequently found to occur in pancreatic juices and a large variety of snake venoms.

AA is usually found at the *sn*-2 position of membrane phospholipids (PE and PC) and can be released by PLA_2. This free AA serves as a precursor for the biosynthesis of the pro-inflammatory lipid mediators (eicosanoids), (Dennis, 1987; Smith, 1989). Phospholipids, the building blocks of biological membranes are constantly metabolised and PLA_2 plays a role in this. PLA_2 is also involved in membrane remodelling through a series of deacylation/reacylation reactions (Van Den Bosch, 1980). PLA_2 also has a membrane protecting role, preventing oxidative damage by preferentially removing peroxidised fatty acids from membrane phospholipids (Van Kuijk *et al.*, 1987).

Characterization has focused mainly on the extracellular PLA_2 enzymes due to their ubiquitous nature and high concentrations found in pancreatic juices and snake venoms

(Verheij *et al.*, 1984; Waite, 1987; Glaser *et al.*, 1993; Dennis, 1994). Traditionally, these extracellular enzymes were divided into three main groups based on their primary amino acid sequence and the positioning of disulphide bridges. The PLA_2 enzymes isolated from the snake venoms of the *Elapidae* and *Hydrophidae* species are classified as group I enzymes. Group II includes those PLA_2 enzymes isolated from the venoms of the *Viperidae* and *Crotalidae* species and those isolated from bee venom belong to group III (Heinrikson *et al.*, 1977; Davidson & Dennis, 1990; Dennis, 1994).

All of these PLA_2 enzymes have been isolated as extracellular enzymes and they share many common traits such as a striking homology in both their amino acid sequence (Verheij *et al.*, 1981; Dennis, 1983) and X-ray crystal structures (Renetseder *et al.*, 1988; Scott *et al.*, 1990 & 1991; White *et al.*, 1990a). These enzymes are small, have a molecular mass of about 14 kDa and differ in their states of aggregation. They are optimally active in the neutral to alkaline pH range. Ca^{2+} is required in the mM range both for catalysis and substrate binding. They are unusually stable due in part to their high disulphide bond content (seven). The catalytic mechanism by which these enzymes hydrolyse the phospholipid substrate has been well studied (Scott *et al.*, 1990 & 1991; Verheij & Dijkstra, 1994). It involves the positioning of His-48 and Asp-99 to form an active Asp-His couple. This couple polarises a bound water molecule which then attacks the carbonyl group and the Ca^{2+} ion stabilises this transition. These extracellular PLA_2 enzymes are optimally active when the phospholipid substrate is part of an interface such as a membrane. Generally these enzymes do not display any selectivity for the fatty acid present in the *sn*-2 position of the phospholipid substrate. In the past few years numerous PLA_2 enzymes have been identified, all essentially

have the same function but do not fit into this traditional categorization. In many cases several of these enzymes are present in the same cell (Marshall & Roshak, 1993). Recently a Ca^{2+} independent, 85 kDa cytosolic phospholipase A_2 ($cPLA_2$) was purified and cloned from human monocytic U937 cells (Kramer *et al.*, 1991; Sharp *et al.*, 1991; for review see Clark *et al.*, 1995). This $cPLA_2$ bears no sequence homology with the extracellular PLA_2s. It appears to be highly selective for phospholipid substrates containing AA at the *sn*-2 position of phospholipids. Another group of calcium independent phospholipase A_2 enzymes ($iPLA_2$) have recently been identified. They have been shown to exist in the brush-border membranes of the small intestine, lysosomes and a wide variety of animal tissues such as the lung, mycoardium and alveolar macrophages (for review see Ackermann & Dennis, 1995) The difficulty of purifying these $iPLA_2$ has hampered their characterization.

1.4.2 The type I secretory PLA_2 ($sPLA_2$-I)

The mammalian pancreatic PLA_2 belongs to the type I PLA_2 class and has been well characterised (for review see Waite, 1987). This enzyme is unique in that it is synthesised and stored as a soluble zymogen or proenzyme in pancreatic acinar cells. Upon stimulation this proenzyme is secreted into the duodenum and converted into its active form through tryptic proteolysis of the N-terminal extra peptide (Nishijima *et al.*, 1983). Once activated it plays a central role in the digestion of dietary phospholipids.

This pancreatic type I PLA_2 enzyme has also been located in a variety of tissues with

no digestive function such as the rat lung, spleen and stomach (Tojo *et al.*, 1988; Sakata *et al.*, 1989; Yasuda *et al.*, 1990) and also in human lung (Seilhamer *et al.*, 1986). The exact function of this extrapancreatic type I PLA_2 enzyme is unclear.

There have been reports suggesting that it may function in a similar manner as a cytokine/growth factor. The type I PLA_2 enzyme can regulate cell proliferation in the Swiss 3T3 fibroblast cell line (Arita *et al.*, 1991). Cell proliferation has also been reported in rat cells such as vascular smooth muscle cells, vascular endothelial cells, synovial cells and chondrocytes (Hanasaki & Arita, 1992) The type I PLA_2 also exhibits a contractile effect in isolated guinea pig lung parenchyma (Sommers *et al.*, 1992; Kanemasa *et al.*, 1992). These physiological effects are thought to be mediated in part through the existence of high affinity type I PLA_2 binding sites.

To date two types of high affinity receptors for these type I PLA_2 enzymes have been characterised. The first type of receptor was initially identified in rat brain (Lambeau *et al.*, 1989). It recognised both the neurotoxic PLA_2 isolated from the Taipan snake venom and the bee venom PLA_2 with high affinity. The porcine pancreatic type I PLA_2 displayed very low affinity for this receptor. A second type of receptor was initially identified in rabbit skeletal muscle by Lambeau *et al.* (1990). This receptor binds both porcine pancreatic type I PLA_2 and the human inflammatory type II PLA_2 with K_d values of 10 and 0.8 nM respectively (Lambeau *et al.*, 1994). A receptor for the type I PLA_2 was also characterised in the Swiss 3T3 fibroblast cell line (Arita *et al.*, 1991). The porcine pancreatic type I PLA_2 displayed high affinity (K_d value of 1.58 nM) whereas the type II PLA_2 isolated from rat and rabbit platelets displayed much lower

affinity. Similar observations were made by Hanasaki & Arita (1992) in rat vascular smooth muscle. The characterised receptor displayed high affinity for the porcine pancreatic type I PLA_2 whereas the type II PLA_2 displayed much lower affinity.

The type I PLA_2 receptors in rabbit (Lambeau *et al.*, 1994), bovine (Ishizaki *et al.*, 1994) and human (Ancian *et al.*, 1995) species have recently been cloned. These cloned receptors are homologous to the macrophage mannose receptor (Taylor *et al.*, 1990) and the DEC-205 receptor recently cloned in dendritic cells (Jiang *et al.*, 1995). All of these receptors share the same structural organisation composed of several distinct domains. These $sPLA_2$ 180-kDa receptors consist of a type I transmembrane protein, which has a large extracellular region composed of an N-terminal cysteine-rich region and a fibronectin type II domain with eight or ten repeats of a carbohydrate recognition domain, followed by a transmembrane domain and a short intracellular C-terminal region. These proteins mediate adsorptive endocytosis.

1.4.3 The type II secretory PLA_2 ($sPLA_2$-II)

In contrast to the type I PLA_2 the intracellular and extracellular type II PLA_2 enzymes have not been as well characterised. They have been found associated with several cells and tissues. Their presence in inflammatory fluids, tissue exudates or serum have implicated a putative role in inflammation (for review see Wong & Dennis, 1990; Pruzanski *et al.*, 1993).

The type II PLA_2 purified from human platelets and human synovial fluid has been

cloned and the complete amino acid sequence determined (Kramer *et al.*, 1989; Seilhamer *et al.*, 1989). Unlike the type I PLA_2, these enzymes contain a 20 amino acid extension preceding the N-terminus resembling a signal sequence. These enzymes appear to be highly conserved between human, porcine and rat displaying an overall homology of 79%. Complete homology was observed for both catalytic site and disulphide bond content. There was no substrate selectivity for fatty acids at the *sn*-2 position of the phospholipid, but preference was displayed for the phospholipid class containing ethanolamine or serine (Kudo *et al.*, 1993).

High levels of the soluble type II PLA_2 have been detected in animal models of inflammation. Vadas & Hay (1982) reported elevated levels of a circulating PLA_2 in rabbits with endotoxin shock. Chang *et al.* (1987a) observed that the PLA_2 purified from the peritoneal exudate of caseinate treated rats was similar to that secreted from rat platelets. Similarly, local and circulating levels of PLA_2 are elevated during infections, inflammatory diseases and injury in humans (Vadas & Pruzanski, 1986). Pruzanski *et al.* (1985) observed high levels of soluble PLA_2 activity both in the sera and synovial fluids isolated from patients with rheumatoid arthritis or osteoarthritis. Purification of this enzyme revealed a dissimilarity with the porcine pancreatic type I PLA_2 (Stefanski *et al.*, 1986). Green *et al.* (1991) also found elevated levels of circulating PLA_2 in patients with sepsis. This inflammatory enzyme was both immunologically and chemically indistinguishable from that associated with rheumatoid arthritis. The source of this PLA_2 is thought to include a number of inflammatory cell types such as neutrophils (Wright *et al.*, 1990), platelets (Kramer *et al.*, 1989) and mast cells (Murakami *et al.*, 1992a).

This type II PLA_2 is an inducible enzyme and arises in response to inflammatory stimuli. Nakano *et al.* (1990) reported that the inflammatory mediators, IL-1 and tumor necrosis factor (TNF) as well as the bacterial lipopolysaccharide (LPS), increased type II PLA_2 mRNA levels in cultured rat vascular smooth muscle cells. This in turn led to an enhanced enzyme secretion from the cells. They also observed that agents which increase cAMP levels (forskolin, isobutylmethylxanthine and dibutyryl cAMP) resulted in increased type II PLA_2 mRNA levels. IL-1, TNF and LPS, had no effect on the cAMP levels. These observations demonstrated that induction of the type II PLA_2 involved a cytokine response element and/or a cAMP response element. Crowl *et al.* (1991) also reported that type II PLA_2 was expressed and secreted from human hepatoma cells in response to the inflammatory mediators IL-6, IL-1 and TNF. IL-6 was the most potent inducer and a synergistic effect was observed both with IL-1 or TNF. Recombinant IL-1 beta (rIL-1β) was found to increase type II PLA_2 mRNA levels in rabbit articular chondrocytes (Kerr *et al.*, 1989). These increased mRNA levels correlated with an increase both in enzyme activity and LTB_4 production over a 20 hour period. In rat cultured astrocytes IL-1, TNF and LPS enhanced type II PLA_2 gene expression and secretion (Oka & Arita, 1991). Dexamethasone suppressed this PLA_2 gene expression induced by LPS but not that of TNF. The time course of PGE_2 formation was similar to that of PLA_2 production when cells were stimulated with LPS. Treatment of rat glomerular mesangial cells with IL-1β, TNF or forskolin stimulated both the synthesis and secretion of type II PLA_2 and PGE_2 (Schalkwijk *et al.*, 1991). Pretreatment of these cells with dexamethasone suppressed the IL-1β, TNF and forskolin induced effects.

The exogenous addition of type II PLA_2 failed to elicit any response in intact cells such as HL-60 granuloctes, mast cells and endothelial cells. However, if cells were activated in the presence of the type II PLA_2 enzyme an enhancement of eicosanoid production was usually observed. Hara *et al.* (1991) reported that both rat and human type II PLA_2 augmented PGE_2 production from A23187 activated HL-60 granuloctes. There was no significant increase observed in PGE_2 production in unstimulated cells. Murakami *et al.* (1991a) also observed that exogenously added type II PLA_2 augmented PGD_2 production in IgE-antigen-primed rat peritoneal mast cells. Similar observations were made with human umbilical endothelial cells (HUVEC) (Murakami *et al.*, 1993b) where exogenously added type II PLA_2 augmented PGI_2 production from TNF activated cells. These observations would suggest that inflammatory conditions are required for the type II PLA_2 to exhibit any effect on the inflammatory response through the generation of AA metabolites. The exact mechanism(s) involved in the action of the type II PLA_2 have yet to be determined.

1.5 Aims of the Study

The type II $sPLA_2$ is present in inflammatory fluids and in inflammatory tissue exudates. Mast cells, in addition to platelets, neutrophils and macrophages, rapidly release this $sPLA_2$ when activated. This extracellular $sPLA_2$ may provide free AA for eicosanoid synthesis by the range of cells associated with the inflammatory condition. In addition, $sPLA_2$-II may provide some phospholipid metabolite which may in turn augment the inflammatory response. The association of elevated levels of the type II $sPLA_2$ enzyme with inflammatory conditions suggested that it may also exert an affect on mast cells. Therefore, one of the aims of this study was to investigate the effect of the type II $sPLA_2$ on mast cell reactivity. Studies in the early 80's showed the involvement of PLA_2 in mast cell degranulation and AA metabolites were proposed to be responsible. These reports were controversial and the recent development of more specific inhibitors for the enzymes involved in the AA metabolic pathway will help clarify this. The effect of these newly developed agents on histamine release from mast cells were also investigated. In addition, the type I $sPLA_2$ was also included in this study as these early observations employed the type I $sPLA_2$ isolated from the porcine pancreas. The responsiveness of mast cells isolated from the rat peritoneum, human lung and guinea pig lung to the $sPLA_2$ enzymes was also examined in view of the known heterogeneity of mast cells throughout the body.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Common chemicals, solvents and buffer components were supplied by Sigma Chemical Co., U.S.A.; BDH Chemicals Ltd., Poole, UK; Mallinckrodt, Kentucky, U.S.A. and Merck, Germany. All aqueous solutions were made up in water prepared with a Millipore milli-Q system.

Phospholipase A_2 enzymes

Phospholipase A_2 (PLA_2) enzymes with defined specific activities, purified from *Naja naja* venom ($sPLA_2$-I) (lot no. 14H7030) and *Crotalus altrox* venom ($sPLA_2$-II) (lot no. 14H9544) were obtained from Sigma. Stock solutions (1,000 U ml^{-1}) were prepared with sterile water, stored in aliquots at -20°C and subsequent dilutions made with Hepes buffer.

Phospholipase A_2 inhibitors

p-Bromophenacyl bromide (*p*-BPB) (lot no. 102H2626), aristolochic acid (lot no. 128F0446) and mepacrine (quinacrine) (lot no. 62H3279) were obtained from Sigma. Stock solutions (10 mM) for *p*-BPB and aristolochic acid were prepared daily with dimethyl sulfoxide (DMSO) and subsequent dilutions made with Hepes buffer. The stock solution (10 mM) for quinacrine was prepared daily with Hepes buffer. 12-deacetyl-12-epi-scalaradial (12-epi-scalaradial), (lot no. M6278) was obtained from BIOMOL Research Laboratories, U.S.A. The stock solution (1 mM) was prepared in

ethanol, stored at -20°C and subsequent dilutions made with Hepes buffer. The cytosolic PLA_2 ($cPLA_2$) inhibitor methyl arachidonyl fluorophosphonate (MAFP), (lot no. 22451913) was obtained from Cayman Chemical Company, U.S.A. The stock solution (5 mg ml^{-1}) was supplied in methyl acetate and stored at -70°C. Dilutions were made with Hepes buffer.

Cyclo-oxygenase inhibitor

Flurbiprofen (lot no. 821885f) was obtained from Cayman Chemical Company. The stock solution (10 mM) was prepared daily with ethanol and subsequent dilutions made with Hepes buffer.

Lipoxygenase inhibitor

Zileuton (A64077) was a generous gift from Abbott Laboratories, UK. The stock solution (10 mM) was made up with DMSO and subsequent dilutions made with Hepes buffer.

Antimycin A

Antimycin A (lot no. 102H4029) was obtained from Sigma. The stock solution (2 mM) was prepared daily with DMSO and subsequent dilutions made with Hepes buffer.

2.2 Buffers

Except where otherwise stated all experiments were performed using Hepes buffered Tyrode's solution pH 7.4, (Hepes buffer) of the following composition: NaCl (137 mM), glucose (5.6 mM), N-2-hydroxyethyl piperazine-N'-2-ethane sulphonic acid (Hepes) (10 mM), KCl (2.7 mM), NaH_2PO_4 (0.4 mM). $CaCl_2$ (1 mM) and $MgCl_2$ (1 mM).

The following buffers were also used throughout this study:

Hepes buffer supplemented with 1 mg ml^{-1} of bovine serum albumin (BSA). (Hepes buffer + BSA).

Hepes buffer supplemented with 10% foetal calf serum (FCS) (Gibco). (Hepes buffer + FCS).

Percoll stock solution: 9 parts of Percoll and 1 part of 10x calcium free Hepes buffer.

Percoll discontinuous gradients were prepared by layering 100%, 80%, 70%, 60%, 50% and 40% Percoll solutions (1 ml) consecutively. Percoll solutions were prepared by dilution with calcium free Hepes buffer.

Isotonic shock solution pH 7.4 consisting of NH_4Cl (0.155 M) and $KHCO_3$ (0.01 M).

Buffers for investigating the requirement of metabolic energy:

Glucose free Hepes buffer (GF Hepes buffer).

GF Hepes buffer supplemented with 2x glucose (11.2 mM) (2x Glucose).

GF Hepes buffer supplemented with 2x antimycin A (0.002 mM) (2x antimycin A).

Buffers for investigating the effects of extracellular calcium:

Calcium and magnesium free Hepes buffered Tyrode's (CMF Hepes buffer).

CMF Hepes buffer supplemented with 2x calcium (2 mM $CaCl_2$) (2x calcium).

CMF Hepes buffer supplemented with 2x EDTA (0.2 mM EDTA) (2x EDTA).

Buffers for the prostaglandin D_2 assay:

Assay buffer: 0.1 M phosphate, pH 7.4 containing NaCl (0.4 M), EDTA (1 mM), BSA (0.1%) and sodium azide (0.01%) (Cayman Chemical Company. U.S.A.). Packet contents were dissolved in 500 ml of Millipore water and stored at 4°C.

Wash buffer: 0.01 M phosphate, pH 7.4 containing Tween-20 (0.05%) (Cayman Chemical Company. U.S.A.). Packet contents were dissolved in 500 ml of Millipore water and stored at 4°C.

Buffer for animal sensitization:

0.01 M phosphate buffered saline (PBS), pH 7.2 containing KCl (2.86 mM), KH_2PO_4 (1.47 mM), NaCl (137 mM) and Na_2HPO_3 (8.09 mM).

2.3 Secretory stimuli

Sheep anti-rat IgE serum, (anti-rat IgE) (product no. 64-352) was obtained from ICN, Costa Mesa, California. Goat anti-human IgE serum, (anti-human IgE) (product no. I-0632) was obtained from Sigma. The lyophilized powder was reconstituted with sterile water (2 ml) and aliquots stored at -20°C.

Chicken egg albumin, (OVA) (grade V, ovalbumin) (lot no. 14H7035) was obtained from Sigma. The powder was stored at 4°C. Stock solutions (1 mg ml^{-1}) were prepared daily with Hepes buffer.

Compound 48/80, (lot no. 53H0006) was obtained from Sigma. The powder was stored at -20°C. Stock solutions (1 mg ml^{-1}) were prepared daily with Hepes buffer and stored at 4°C.

The calcium ionophore A23187 (lot no. 114H4025) was obtained from Sigma. Stock solutions (10 mM) were prepared with DMSO, stored in aliquots at -20°C and subsequent dilutions made with Hepes buffer.

2.4 Source of mast cells

Rat peritoneal mast cells were obtained from male Sprague Dawley rats (body weight 250-350 g). Guinea pig lung mast cells were obtained from male and female Dunkin Hartley guinea pigs (body weight 300-500 g). These animals were obtained from the animal house located in the Basic Medical Science Building, The Chinese University of Hong Kong.

Human lung mast cells were isolated from human lung tissue obtained from cancer patients undergoing resection of the lung at the Prince of Wales Hospital, Shatin. Macroscopically healthy areas of a lung lobe, judged normal by a pathologist, were placed in Hepes buffer at 4-10°C for transport to the laboratory. Human lung mast

cells were isolated from the tissue within 24 hours.

2.4.1 Sensitization of animals

For studies on immunologically induced histamine release, cells were isolated from animals that had previously been exposed to allergen (ovalbumin). Both rat and guinea pig were sensitized according to the following procedures.

Rat sensitization: Male rats (body weight 200-250g) were given an intraperitoneal injection of PBS (0.5 ml) containing OVA (1 mg) and $Al(OH)_3$ (100 mg). The adjuvant was added to the allergen solution, with constant stirring 30 min before injection. Animals were sacrificed 3 weeks later.

Guinea pig sensitization: Male and female guinea pigs were sensitized using an emulsified solution of OVA and Freund's complete adjuvant. The solution of allergen and adjuvant was expelled repeatedly through two Leur Lock syringes connected via a double hubbed needle to form an emulsion. 1 ml of the emulsion (50 mg OVA in 0.5 ml of saline and 0.5 ml of Freund's complete adjuvant) was injected intraperitoneally into each guinea pig. Animals were sacrificed 3 weeks later.

2.4.2 Isolation of rat peritoneal mast cells

The rat was first anaesthetized with ether and sacrificed by cervical dislocation followed by exsanguination. Hepes buffer (20 ml) containing heparin (5 units ml^{-1})

was injected intraperitoneally. The abdomen was gently massaged for 2 min and cut open along the midline. Peritoneal cells were recovered using a plastic Pasteur pipette and collected into polystyrene tubes chilled on ice. The cells were pelleted by centrifugation (180xg, 4°C, 5 min). Non-purified cells were washed twice in Hepes buffer and used for functional studies without further treatment. Samples heavily contaminated with blood were discarded. The cell pellet usually contained 7.6 ± 0.6% mast cells.

2.4.3 Purification of rat peritoneal mast cells

Cells to be purified were isolated as before (section 2.4.2). The cells were washed once in Hepes buffer + BSA, and the pellet resuspended in 1 ml of Hepes buffer + BSA (Mackay & Pearce, 1992). The cells were mixed with 4 ml of a Percoll stock solution and Hepes buffer + BSA (1 ml) was layered on top to produce an interface. This was centrifuged at 140xg for 25 min at 4°C. The resulting cell pellet usually contained 91.9 ± 0.8% mast cells. This was washed twice in Hepes buffer + BSA and twice in Hepes buffer before use for functional studies.

2.4.4 Isolation of human lung mast cells

Human lung tissue was dissected free of pleura, large bronchi and blood vessels. The tissue was washed twice in Hepes buffer and minced finely with scissors into approximately 1 mm^3 fragments. These fragments were washed extensively with Hepes buffer + BSA to remove blood and mucus. The chopped tissue was then

incubated at 37°C for 60 min with gentle agitation in a solution of collagenase (Sigma type 1A, 80 Units ml^{-1}) in Hepes buffer + BSA. At the end of the incubation, the mixture was filtered through gauze, to remove undisrupted tissue. The isolated cells were recovered by centrifugation (200xg, 25°C, 5 min). Cells were washed twice in Hepes buffer + BSA and once in Hepes buffer. Cells were finally resuspended in Hepes buffer + FCS and stored temporarily at 4°C. The remaining undisrupted tissue was subjected to a second incubation as described above. At the end of this incubation, the tissue was disrupted by expression through a syringe. The resulting suspension was filtered and the cells recovered, were washed as before. Cells from both incubations were pooled and kept at 4°C in Hepes buffer + FCS. The cell suspension was washed twice in Hepes buffer before use for functional studies. The % mast cells present was usually 4.7 ± 0.7% of the total nucleated cells.

2.4.5 Partial purification of human lung mast cells

The method employed was an adaptation of the procedure described by Schulman *et al.* (1988) for the purification of human lung mast cells. Cells to be purified were washed once with Hepes buffer. The pellet was resuspended in 3 ml of an isotonic shock solution and kept on ice for 5 min. The reaction was stopped by the addition of Hepes buffer + BSA (20 ml). Cells were pelleted by centrifugation (200xg, 5 min, 4°C), and resuspended in 1 ml of Hepes buffer + BSA. The cell suspension was layered on top of a Percoll discontinuous gradient and centrifuged at 400xg for 20 min at 4°C. Cells from each interface were collected and mast cells were generally found at the 50%, 60% and 70% interfaces. Fractions rich in mast cells were pooled

together and washed twice in Hepes buffer to remove any remaining Percoll. Cells were stored in Hepes buffer + BSA and kept at 4°C until use for functional studies.

2.4.6 Isolation of guinea pig lung mast cells

Animals were sacrificed by cervical dislocation followed by exsanguination. Lungs were removed from the thoracic cavity. Lung mast cells were recovered as described previously (2.4.4). The incubation buffer contained collagenase at a concentration of 160 Units ml^{-1}. The % mast cells present was usually 3.9 $\pm$ 0.8% of the total nucleated cells.

2.5 General procedure for studying histamine release from isolated mast cells

Both purified and non-purified cells were prewarmed for 5 min at 37°C in a shaking water bath. Aliquots of cells (500 μL for rat peritoneal mast cells (RPMC), 360 μL for human (HLMC) and guinea pig lung mast cells (GPLMC)) were added to prepared polystyrene tubes containing either the buffer alone (500 μL for RPMC, 40 μL for HLMC and GPLMC) or buffer and stimulus. All experiments using RPMC included duplicate tubes for each sample. The histamine release reaction was allowed to proceed for 10 min for RPMC and HLMC, and 20 min for GPLMC. The reaction was stopped by placing the tubes in an ice cold water bath, followed by the addition of ice cold Hepes buffer (2 ml for RPMC, 600 μL for HLMC and GPLMC). Cells were immediately separated from the supernatants by centrifugation (180xg, 4°C, 5 min). The remaining cell pellets were resuspended in Hepes buffer (3 ml for RPMC, 1 ml

for HLMC and GPLMC) and boiled for 15 min to release the residual histamine. Samples were either assayed immediately or stored at -20°C. The following modifications of this procedure were adapted throughout this study.

2.5.1 Procedure for investigating the effect of the $sPLA_2$ enzymes on the spontaneous histamine release from isolated mast cells

Aliquots of cells were added to prepared tubes containing the $sPLA_2$ enzymes (50 μL for RPMC, 40 μL for HLMC and GPLMC). Cells were incubated with the $sPLA_2$ enzymes for various time periods: RPMC (10, 20 and 30 min), HLMC (10 and 20 min) and GPLMC (20 and 30 min). The reaction was stopped by the addition of appropriate volumes of ice cold Hepes buffer. Thereafter the procedure was as described above (section 2.5). The kinetics of the spontaneous histamine release induced by $sPLA_2$-I (0.1 and 1.0 U ml^{-1}), on purified RPMC over a time period of 1 to 40 min were also examined.

2.5.2 Procedure for investigating the effect of heat treated $sPLA_2$-I on histamine release from isolated RPMC

The $sPLA_2$-I stock solution was boiled for 30 min and allowed to cool at room temperature. This was then incubated with RPMC for 10 min and the procedure described before was followed (section 2.5). Unheated $sPLA_2$-I was used as the control.

2.5.3 Procedure for investigating the effect of antimycin A on histamine release induced by $sPLA_2$-I on isolated RPMC

RPMC were recovered as described previously (section 2.4.2). Non-purified cells were washed and resuspended in GF Hepes buffer. Aliquots of cells (500 μL) were added to prepared tubes containing 500 μL of the following buffers; 2x Glucose, GF Hepes buffer and 2x antimycin A. Cells were incubated for 20 min before the addition of $sPLA_2$-I or compound 48/80 (25 μL). The histamine release reaction was allowed to proceed for 10 min. The reaction was stopped by the addition of ice cold GF Hepes buffer and the procedure described in section 2.5 was followed.

2.5.4 Procedure for investigating the effect of extracellular calcium on histamine release induced by $sPLA_2$-I on isolated RPMC

Purified RPMC were recovered as described previously (2.4.2), and resuspended in CMF Hepes buffer. Aliquots of cells (500 μL) were added to prepared tubes containing 500 μL of the following buffers; 2x calcium, CMF Hepes buffer and 2x EDTA. Cells were incubated for 10 min in the appropriate tubes at 37°C. Aliquots of $sPLA_2$-I (25 μL) were added and the reaction was terminated by the addition of ice cold CMF Hepes buffer. Thereafter, the procedure described in section 2.5 was followed.

2.5.5 Procedure for investigating the effects of the $sPLA_2$ enzymes on activated mast cells

Aliquots of cells were added to prepared tubes containing the $sPLA_2$ enzymes. Cells were preincubated with the $sPLA_2$ enzymes for 10 min at 37°C. Mast cell secretagogues (anti-IgE, ovalbumin, compound 48/80 and A23187) (25 μL) were added to activate histamine secretion and the reaction allowed to proceed, for 10 min for RMPC or HLMC and 20 min for GPLMC. The reaction was stopped as described in section 2.5. Controls containing cells with the $sPLA_2$ enzymes or secretagogue alone were also included.

2.5.6 Procedure for investigating the effect of preincubation time on $sPLA_2$ induced histamine release from immunologically activated RPMC

Aliquots of RPMC were added to prepared tubes containing the $sPLA_2$ enzymes and preincubated for 1, 5 and 10 min at 37°C. Anti-rat IgE (25 μL) was then added to activate histamine secretion and the reaction allowed to proceed for 5 and 10 min. The reaction was stopped as described in section 2.5. Controls containing cells with the $sPLA_2$ enzymes or secretagogue alone were also included.

2.5.7 Procedure for investigating the effects of the PLA_2 inhibitors

The $sPLA_2$ enzymes were preincubated with the various PLA_2 inhibitors for 30 min at 37°C. Cells were then added and incubated for a further 20 min. Thereafter the

procedure described in section 2.5 was followed. Controls containing cells with the inhibitors or the $sPLA_2$ enzymes were also included. The inhibitors were dissolved in Hepes buffer, DMSO, ethanol or methyl acetate and controls for these vehicles were included in all experiments. When studying the effect of these inhibitors on immunologically activated mast cells, the $sPLA_2$ enzymes were preincubated with the inhibitors for 30 min at 37°C. Aliquots of cells were then added and incubated for 10 min. Anti-IgE (25 μL) were then added to stimulate secretion for a further 10 min. The reaction was terminated as before (2.5). Controls containing cells with the inhibitors, $sPLA_2$ enzymes or secretagogue alone were also included.

2.5.8 Procedure for investigating the effects of the cyclo-oxygenase and lipoxygenase inhibitor

Aliquots of purified RPMC were preincubated with flurbiprofen or zileuton for 15 min at 37°C. The $sPLA_2$ enzymes (25 μL) were then added and incubated for a further 20 min. Thereafter the procedure described in section 2.5 was followed. Controls containing cells with the inhibitors or $sPLA_2$ enzymes alone were also included. When studying the effect of these inhibitors on immunologically activated mast cells, cells were preincubated for 15 min. The $sPLA_2$ enzymes (25 μL) were then added and incubated for 10 min. Anti-rat IgE (25 μL) was then added to stimulate secretion for a further 10 min. The reaction was terminated as before (2.5). Controls containing cells with the inhibitors, $sPLA_2$ enzymes or secretagogue alone were also included.

2.6 Histamine assay

The histamine assay was first described by Shore *et al.* (1959) and is based on the reaction between histamine and *o*-phthaldialdehyde (OPT) under alkaline conditions. The resultant condensation product is highly fluorescent and can be measured using a spectrofluorimeter.

Samples from RPMC were measured for histamine using the simple condensation assay without any extraction steps (Loeffler *et al.*, 1971). Briefly, samples (3 ml) were treated with NaOH (1M, 400 μL), followed by the addition of OPT (100 μL, 1mg ml^{-1} in methanol) and samples immediately mixed. The reaction was stopped 4 min later by the addition of HCl (3M, 200 μL). Fluorescence measurements were obtained at an excitation wavelength of 360 nm with an emission wavelength of 450 nm. Bandwidths were set at 5 and 10 nm respectively. Measurements were performed using a Hitachi Fluorescence Spectrophotometer F-4010 at room temperature.

Samples from HLMC and GPLMC were measured for histamine using an automated fluorometric method (Ennis, 1991). Samples were treated with perchloric acid (0.4N) to precipitate out any protein present and centrifuged (250xg, 25°C, 25 min). Following introduction into the autoanalyzer, samples were made alkaline and histamine was extracted into butanol. The organic phase was separated and washed once in a less alkaline medium, made less polar by the addition of heptane and the histamine back-extracted into dilute sulphuric acid. The amine was allowed to react with OPT under alkaline conditions and the adduct generated was stabilized by

acidification. Fluorescence was detected with a fluorometer and recorded on a chart recorder. The automated analyzer was a BRAN + LUEBBE AutoAnalyzer II and was used in conjunction with the AACE evaluation software which provided comprehensive tools to process and evaluate the data collected.

Histamine was measured in both the supernatant and cell pellet fractions. Histamine release was expressed as a percentage of the total cellular histamine content:

$$\% \text{ Histamine release } (H_R) = 100 \times (H_S / (H_S + H_C))$$

where H_S represents the amount of histamine in the supernatant and H_C the corresponding amount remaining in the cell pellet. The percentage inhibition of histamine release was calculated as follows:

$$\% \text{ Histamine inhibition} = 100 \times (H_E - (H_{E+P} - H_P)) / H_E$$

where H_E represents the amount of histamine released with the secretagogue alone, H_{E+P} represents the amount of histamine released by the secretagogue in the presence of the $sPLA_2$ enzymes and H_P represents amount of histamine released by the $sPLA_2$ enzyme alone. In each experiment all values were corrected for the spontaneous release of histamine occurring in the absence of any stimulus.

2.7 Measurement of Prostaglandin D_2

2.7.1 Prostaglandin D_2 methoxime enzyme immunoassay kit

Prostaglandin D_2 (PGD_2) was measured with a prostaglandin D_2 methoxime (PGD_2 MOX) enzyme immunoassay kit which was obtained from Cayman Chemical

Company. U.S.A. and stored at -20°C. Procedures were carried out according to the instructions in the assay kit.

2.7.2 The Prostaglandin D_2 assay

PGD_2 itself is a relatively unstable eicosanoid and in this assay is chemically converted to a more stable compound. Briefly, samples were treated with methoxamine hydrochloride which converts the unstable PGD_2 into a stable methoxime derivative PGD_2-MOX. The assay is based on the competition between free PGD_2-MOX and a fixed amount of acetylcholinesterase labelled PGD_2-MOX, for a limited number of binding sites on a PGD_2-MOX specific antibody. Thus, with fixed amounts of antibody and enzyme linked PGD_2-MOX, the amount of acetylcholinesterase labelled PGD_2-MOX bound by the antibody will be inversely proportional to the amount of free PGD_2-MOX. The antibody PGD_2-MOX (free or acetylcholinesterase labelled) complex binds to a second antibody bound to the plate well. Any unbound compounds are washed away. The amount of acetylcholinesterase labelled PGD_2-MOX bound by the antibody can be determined by adding Ellman's reagent (contains the substrate for acetylcholinesterase) to the well. The intensity of the product has a distinct yellow coloured product and can be determined spectrometrically. This is proportional to the amount of labelled PGD_2-MOX bound to the well.

2.7.3 PGD_2 release from purified rat peritoneal mast cells

Samples were removed from the supernatant fraction of the histamine release reaction

as described in section 2.5. These samples were immediately frozen by immersion in liquid nitrogen, stored at -70°C and assayed within 2 weeks.

2.7.4 Derivatization of PGD_2-MOX

All samples and the PGD_2 standard (dissolved in assay buffer) were converted to the stable methoxime derivative. 100 μL of the oximating reagent (methoxylamine HCl mixed with sodium acetate in a 10% ethanol solution) was added to all polystyrene tubes containing 100 μL of sample or standard. All tubes were heated at 60°C for 30 min. Sample tubes were centrifuged (100xg, 25°C, 5 min) and the supernatant fraction diluted with assay buffer. PGD_2 standards (8 to 1000 pg ml^{-1}) were prepared with assay buffer from the PGD_2-MOX standard. A typical standard curve for PGD_2-MOX is shown in fig 2.1.

2.7.5 PGD_2 assay procedure

100 μL of assay buffer was pipetted into the non-specific binding (NSB) wells and 50 μL into the maximun binding wells (Bo) in duplicate. 50 μL aliquots from each standard and sample were pipetted into appropriate wells in duplicate. 50 μL of the acetylcholinesterase labelled PGD_2-MOX was added into all wells except the blank, followed by 50 μL of antiserum (containing the PGD_2-MOX specific antibody) to all wells except the blank and NSB wells. The plate was covered and incubated overnight at room temperature. After the incubation any unbound reagents were removed by washing (5 times) with wash buffer. The plate was blotted onto tissue paper to ensure

complete removal of any remaining residual liquid. 200 μL of Ellman's reagent was immediately dispensed into all wells. The plate was covered, mixed gently and incubated at room temperature for 90 min in the dark. The absorbance (optical density) was determined in an MR5000/7000 Microplate Reader (Dynatech Laboratories) at 405 nm.

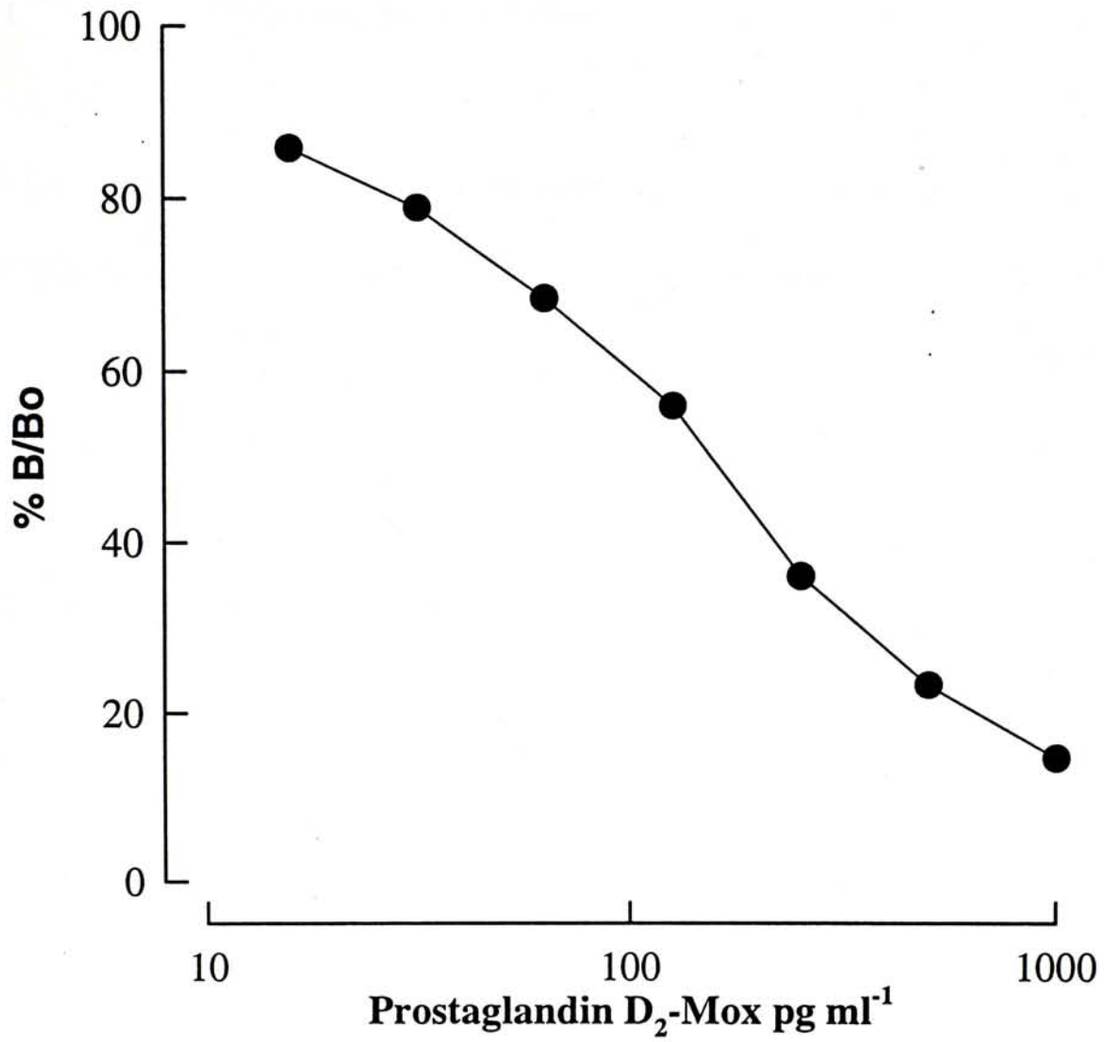

Figure 2.1 Typical standard curve for the PGD_2-MOX enzyme immunoassay

The assay procedure was described in the text (2.7.5). Average optical density (OD) readings for each standard in duplicate was calculated. A standard curve was generated by plotting the percent bound/maximum bound (%B/Bo) as a log function of the PGD_2-MOX concentration. The %B/Bo for each standard and sample was calculated as follows;

%B/Bo = 100 X (standard OD - NSB OD / zero standard OD - NSB OD)

where Bo represents the amount of acetylcholinesterase labelled PGD_2-MOX bound to the antibody in the absence of any standard (zero standard), B represents the amount of acetylcholinesterase labelled PGD_2-MOX bound to the antibody in the presence of a known standard and NSB represents the amount of acetylcholinesterase labelled PGD_2-MOX non specifically bound to the well. All OD readings were corrected for background (blank).

2.8 Measurement of intracellular calcium

2.8.1 Fluorescent calcium indicator fura-2

The fluorescent probe fura-2 was packaged as a 1 mg aliquot of the unstable acetoxymethyl ester form. This aliquot was reconstituted in DMSO to a concentration of 1 mM. This was then aliquoted and stored in tightly sealed micro centrifuge tubes at -20°C.

2.8.2 Cell loading of the calcium indicator

Purified rat peritoneal mast cells were obtained as described in section 2.4.3. Mast cell suspensions were incubated with the acetoxymethyl ester of fura-2 (1 μM) in Hepes buffer + BSA at 37°C for 15 min (Kuno *et al.*, 1993). In all experiments, a control sample of the cell suspension was not incubated with the calcium indicator. These cells were treated similarly and used to measure autofluorescence. Cells were washed twice to remove any extracellular calcium indicator and resuspended in Hepes buffer at a cell density of 1×10^6 cells ml^{-1}. This was then transferred to a quartz cuvette, which contained a magnetic stirrer.

2.8.3 Measurement of fura-2 fluorescence

Measurements of intracellular calcium were performed using a Perkin Elmer Luminescence Spectrometer LS 50B. The cuvette was maintained in a temperature

controlled water jacket at 37°C. Samples were cyclically excited by wavelengths of 340 and 380 nm, with switching at an interval of 0.25 sec. The resulting fluorescence was measured at the emission wavelength of 510 nm and data processed using the LS 50B software package. The bandwidth of the excitation pathway was set at 10 nm, whereas the emission pathway bandwidth was 20 nm. At predetermined time points, with the aid of a Hamilton syringe, the stimulating agent ($sPLA_2$ or anti-rat IgE) was applied to the mast cell suspension. Histamine release for each sample was directly determined by removing 250 μL of the supernatant from the cuvette after the observation period was complete. This was immediately centrifuged and the procedure described in section 2.5 was followed.

The following calibration procedure was used: cells were lysed with digitonin (100 μg ml^{-1}) to generate the maximum fluorescence value (S_{max}) followed by Ca^{2+} chelation with EGTA/Tris (4 mM EGTA), to generate the minimum fluorescence value (S_{min}) (Thomas & Delaville, 1991). Calcium levels were calculated from the ratio of the fluorescence by using the following equation described by Grynkiewicz *et al.* (1985);

$$[Ca^{2+}]_i \text{ (nM)} = K_d (S-S_{min})/(S_{max}-S) \times F_{380,\ free}/F_{380,\ bound}$$

where K_d is the dissociation constant for the fura-2 Ca^{2+} complex and is equivalent to 224 nM at 37°C. S_{min} and S_{max} are the ratios of the fluorescence when fura-2 is in the absence of Ca^{2+} and saturated with Ca^{2+} respectively. S is the experimental ratio and $F_{380,\ free}$ / $F_{380,\ bound}$ is the ratio of fluorescence excited at 380 nm between free and saturating Ca^{2+} conditions. Autofluorescence values were obtained from cells not loaded with the dye by measuring the signal at 340 and 380 nm. Calibrations and autofluorescence measurements were performed daily.

Fluorescence measurements for all samples were made for 1 min before the addition of stimulus. Any increase in the fluorescence intensity following stimulus addition were related to this 1 min basal reference level. In all experiments fluorescence was measured for a sample of cells in the absence of any stimulus. It was observed that the fluorescence intensity increased with the passage of time. Therefore all measurements were corrected for this spontaneous increase in calcium.

2.9 Cell counts

Mast cell number and cell viability was determined for all mast cell populations with a haemocytometer. For an estimation of the percentage viability, 1 part of the cell suspension was stained with 1 part of Trypan blue (0.4 %) for 5 min at 37°C. Dead cells retain this dye and appear dark blue in colour. With regard to mast cell number, 9 parts of the cell suspension were stained with 1 part of Alcian blue. The alcian blue dye contained 1% Alcian blue, 1% tween 20 and 0.9% NaCl in 0.5 M HCl. Staining was allowed to proceed for 5 min at 37°C. Mast cells retain this dye and appear bright blue in colour. Cell counting was performed within 15 min after staining.

2.10 Data analysis

All values are given as the means ± standard error of the mean (SEM) for the number (n) of experiments performed. Differences between groups of data were compared using Student's *t* tests (SIGMA plot). Significance was claimed when $p \leq 0.05$.

CHAPTER 3

EFFECTS OF SPLA$_2$ ON RAT PERITONEAL MAST CELLS

3.1 Introduction

Mast cells and their derived mediators are known to play a central role in allergic reactions. Mediators such as histamine, PGD_2 and a variety of cytokines are released following cell activation. These mediators are known to cause many biological effects such as vasodilation, increased vascular permeability and cellular recruitment. Mast cells are associated with many chronic inflammatory conditions such as inflammatory bowel disease and rheumatoid arthritis. Besides mast cells, increased levels of the type II $sPLA_2$ enzyme have also been detected in the synovial fluid and plasma of patients with these inflammatory diseases. The source of this enzyme is thought to include many inflammatory cells such as neutrophils, macrophages and mast cells. The association of elevated levels of the type II $sPLA_2$ enzyme with inflammatory conditions suggested that it may also affect mast cells. Previous studies in the early 1980's, showed that the $sPLA_2$ purified from the porcine pancreas (classified as a type I $sPLA_2$), caused mast cell secretion and this was blocked by PLA_2 inhibitors. It was proposed that PLA_2 activation was involved in mast cell secretion. Therefore, the aim of this study was to investigate the effects of a type I and type II $sPLA_2$ on mast cell reactivity. The $sPLA_2$ purified from *Naja naja* venom and *Crotalus altrox* venom were chosen as examples of the type I and type II $sPLA_2$ enzymes respectively. Rat peritoneal mast cells were chosen as a model due to their abundance and ease of isolation and purification. Histamine is immediately released from mast cells after activation and was measured with a fluorometric assay as the indicator for mast cell degranulation.

3.2 Methodology

The procedures described in chapter two were applied here

PART 1: Effects of $sPLA_2$-I on unstimulated rat peritoneal mast cells

3.3 Results

3.3.1 Effects of $sPLA_2$-I on the spontaneous histamine release from RPMC

Rat peritoneal mast cells were incubated with $sPLA_2$-I for 10, 20 and 30 min. Histamine release was induced by $sPLA_2$-I (0.5 to 10 U ml^{-1}) in a dose dependent manner (Fig 3.1). Cells incubated for 30 min released significantly more histamine than those incubated for 10 min (0.5 to 5 U ml^{-1}, $p \leq 0.05$). $sPLA_2$-I (5 U ml^{-1}) induced histamine release was 74.72 $\pm$ 1.49% for the 30 min incubation compared to 62.79 $\pm$ 6.33% for the 10 min incubation. Concentrations between 0.5 and 1 U ml^{-1}, incubated for 20 min, released significantly more histamine than the 10 min incubation ($p \leq 0.05$). 0.05 and 0.01 U ml^{-1} of $sPLA_2$-I did not have any effect on the spontaneous histamine release.

To eliminate the chance that any remaining functional cells such as macrophages or neutrophils were influencing the results, mast cells were purified. Similar results were obtained when purified mast cells were incubated with $sPLA_2$-I (0.01 to 10 U ml^{-1}). Histamine release was again observed dose dependently at all three time incubations

(Fig 3.2). Cells incubated for 20 and 30 min significantly released more histamine than those cells incubated for 10 min (1 to 10 U ml^{-1}, $p \leq 0.01$). The maximum histamine release observed was 67.91 $\pm$ 2.31% following a 30 min incubation compared with 48.32 $\pm$ 3.92% for the 10 min incubation. Lower concentrations of $sPLA_2$-I did not have any effect on the spontaneous histamine release.

3.3.2 Time course of $sPLA_2$-I induced histamine release

Fig 3.3 illustrates the time course of $sPLA_2$-I induced histamine release (1 and 0.1 U ml^{-1}). With an $sPLA_2$-I concentration of 1 U ml^{-1}, histamine release was rapid within the first 5 minutes and plateaued after 10 minutes. The maximum histamine release observed was 79.85 $\pm$ 2.69%. Whereas with 0.01 U ml^{-1}, histamine release was slow and plateaued after 30 minutes of incubation, with a maximum release of 60.13 $\pm$ 4.83%.

3.3.3 Effects of heat treated $sPLA_2$-I on the spontaneous histamine release

To further characterise the histamine releasing activity of $sPLA_2$-I, the enzyme preparation was heated at 100°C for 30 min. When cells were incubated with this heated enzyme there was no histamine release observed (Fig 3.4). Indeed the activity of the enzyme was significantly reduced when compared to the unheated enzyme (1 to 10 U ml^{-1}, $p \leq 0.05$).

3.3.4 Effects of antimycin A on $sPLA_2$-I induced histamine release

The removal of glucose from the incubation buffer did not reduce the histamine release induced by $sPLA_2$-I or the classical mast cell secretagogue compound 48/80 (Table 3.1). However substitution with the metabolic inhibitor antimycin A did significantly reduce histamine secretion at all $sPLA_2$-I concentrations tested ($p \leq 0.01$). Antimycin A is an inhibitor of electron transfer between cytochrome b and c in the oxidative phosphorylation pathway (Ahmed *et al.*, 1951). The spontaneous histamine release was comparable in all the incubation media.

3.3.5 Effects of extracellular calcium on $sPLA_2$-I induced histamine release

Histamine release induced by $sPLA_2$-I was significantly reduced in the absence of extracellular calcium (Fig 3.5) (0.01 to 1 U ml^{-1}, $p \leq 0.01$). However, between 1 and 10 U ml^{-1}, histamine release was observed in the absence of extracellular calcium, although it was significantly lower than the corresponding control. The maximum histamine release observed was 66.69 $\pm$ 4.36% (control) compared with 14.55 $\pm$ 1.19% without calcium (10 U ml^{-1}). Histamine release was totally abolished by pretreatment of the cells with the chelating agent EDTA. The spontaneous histamine release was unaffected by all these conditions.

3.3.6 Effects of PLA_2 inhibitors on $sPLA_2$-I induced histamine release

The effects of *p*-Bromophenacyl bromide (*p*-BPB), mepacrine and aristolochic acid on

the histamine releasing activity of $sPLA_2$-I were also studied. *p*-BPB inactivates snake venom PLA_2 by alkylation of the histidine residue found in the catalytic site (Volwerk *et al.*, 1974; Roberts *et al.*, 1977). The anti-malarial agent mepacrine is thought to inhibit PLA_2 hydrolysis by disrupting the lipid water interface required for optimal enzyme activity (Chang *et al.*, 1987b). Aristolochic acid derived from *Aristolochia* plant species inhibits snake venom PLA_2 and human synovial fluid PLA_2 *in vitro* (Vishwanath *et al.*, 1988).

Table 3.2 shows the effect of different pretreatment times with *p*-BPB, mepacrine and aristolochic acid on $sPLA_2$-I induced histamine release. $sPLA_2$-I induced histamine release was significantly reduced by *p*-BPB at all pretreatment times investigated. A significant reduction was also observed with mepacrine and aristolochic acid. These inhibitors caused no significant alteration to the spontaneous histamine release. *p*-BPB was the most potent of the inhibitors tested and this inhibitory effect was further investigated with 30 min being chosen as a suitable pretreatment time.

Pretreatment of $sPLA_2$-I (1 U ml^{-1}) with *p*-BPB, significantly decreased the induced histamine release in a dose dependent manner, at all concentrations examined, except at 0.1 μM (Fig 3.6a). However, with an $sPLA_2$-I concentration of 0.01 U ml^{-1}, histamine release was only significantly reduced with 10 μM of *p*-BPB (Fig 3.6b).

The effects of *p*-BPB on $sPLA_2$-I induced histamine release from purified rat peritoneal mast cells were also examined. A 30 min pretreatment of $sPLA_2$-I (0.1 U ml^{-1}) with *p*-BPB, significantly reduced the induced histamine release at concentrations of 1 and

10 μM (Fig 3.7a). With an $sPLA_2$-I concentration of 0.01 U ml^{-1}, histamine release was only significantly reduced with *p*-BPB concentrations of 1 and 10 μM (Fig 3.7b).

One of the hydrolysis products of heteronemin (a scalarane found in many Indo-Pacific sponges), 12-deacetyl-12-epi-scalaradial (12-epi-scalaradial) has been shown to inhibit the bee venom PLA_2 with an IC_{50} of 0.2 μM (Potts *et al.*, 1992). Pretreatment of $sPLA_2$-I (0.1 U ml^{-1}) with 12-epi-scalaradial significantly decreased the induced histamine release dose dependently (Fig 3.8a). With the lower concentration of $sPLA_2$-I of 0.01 U ml^{-1} the reduction was dose dependent between 0.3 and 1.0 μM (Fig 3.8b).

Methyl arachidonyl fluorophosphonate (MAFP) has been reported to be a potent irreversible inhibitor of $cPLA_2$ (Huang *et al.*, 1994). It was shown to inhibit A23187 induced LTB_4 production in human neutrophils with an IC_{50} of 0.1 μM. This $cPLA_2$ inhibitor did not have any inhibitory effect on the $sPLA_2$-I induced histamine release from purified rat peritoneal mast cells (Fig 3.9a + b).

3.3.7 Effects of $sPLA_2$-I on PGD_2 production

In a limited series of experiments (n = 3), the effects of $sPLA_2$-I on PGD_2 production from purified rat peritoneal mast cells were also examined. Fig 3.10 shows that at the highest concentration tested, 1 U ml^{-1}, there was an appreciable amount of PGD_2 produced (112.25 $\pm$ 31.75 ng/10^6 cells). Lower concentrations of $sPLA_2$-I did not seem to have any effect on PGD_2 production.

3.3.8 Effects of $sPLA_2$-II on the spontaneous histamine release from RPMC

In contrast to the type I $sPLA_2$, when purified rat peritoneal mast cells were incubated with $sPLA_2$-II (20 min), there was generally less than 10% of the total cellular histamine release observed (Fig 3.11). The highest release of about 10% was observed with 1 U ml^{-1}. Similar results were obtained with both mixed and purified rat peritoneal mast cells (Fig 3.12). However, $sPLA_2$-II concentrations greater than 1 U ml^{-1} seemed to have an inhibitory effect on the spontaneous histamine release.

3.3.9 Effects of PLA_2 inhibitors on $sPLA_2$-II induced histamine release from purified RPMC

Fig 3.13a illustrates the effects of a 30 min, *p*-BPB pretreatment on $sPLA_2$-II (1 U ml^{-1}) induced histamine release. 1 and 10 μM of *p*-BPB significantly reduced the low histamine release induced by $sPLA_2$-II itself (10.09 $\pm$ 0.97%). Fig 3.13b illustrates the effects of a 30 min, 12-epi-scalaradial pretreatment on $sPLA_2$-II (1 U ml^{-1}) induced histamine release. 0.6 and 1 μM significantly reduced the low $sPLA_2$-II induced histamine release.

3.3.10 Effects of $sPLA_2$-II on PGD_2 production

Fig 3.14 illustrates the effects of $sPLA_2$-II on PGD_2 production from purified rat peritoneal mast cells. PGD_2 was generated in a concentration dependent manner from cells incubated with $sPLA_2$-II for 20 min. The maximum PGD_2 produced was 41.19

$\pm$ 9.69 ng/10^6 (1 U ml^{-1}) compared with 31.19 $\pm$ 6.73 ng/10^6 cells with anti-rat IgE (1/100).

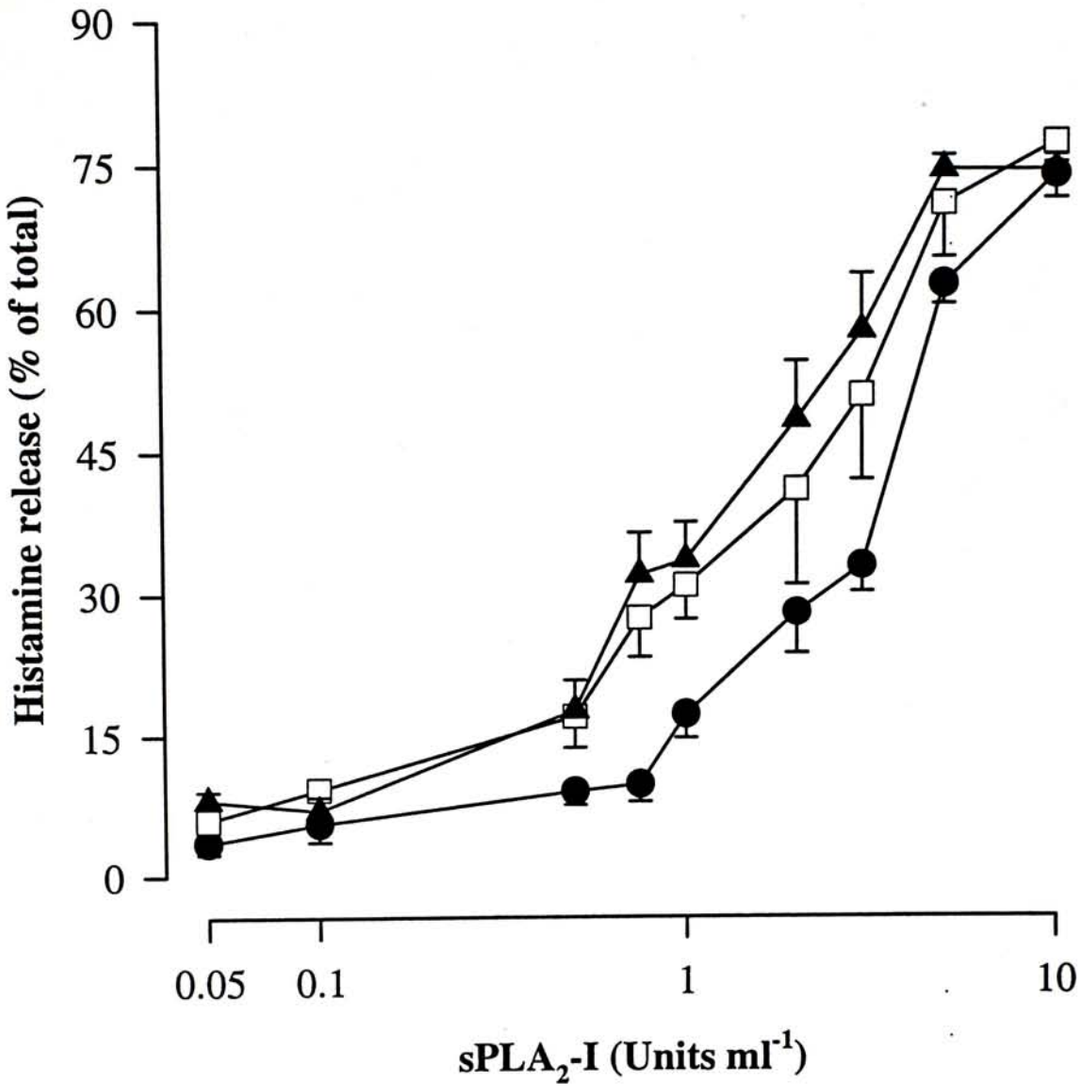

Figure 3.1 Effects of $sPLA_2$-I on the spontaneous histamine release from rat peritoneal mast cells. Cells were incubated with $sPLA_2$-I (0.05 to 10 U ml^{-1}) for 10 (●), 20 (□) and 30 min (▲). The spontaneous histamine release was 7.13 ± 0.69% (10 min), 8.42 ± 0.54% (20 min) and 11.75 ± 0.87% (30 min). Results are given as the means ± SEM for n = 3-8.

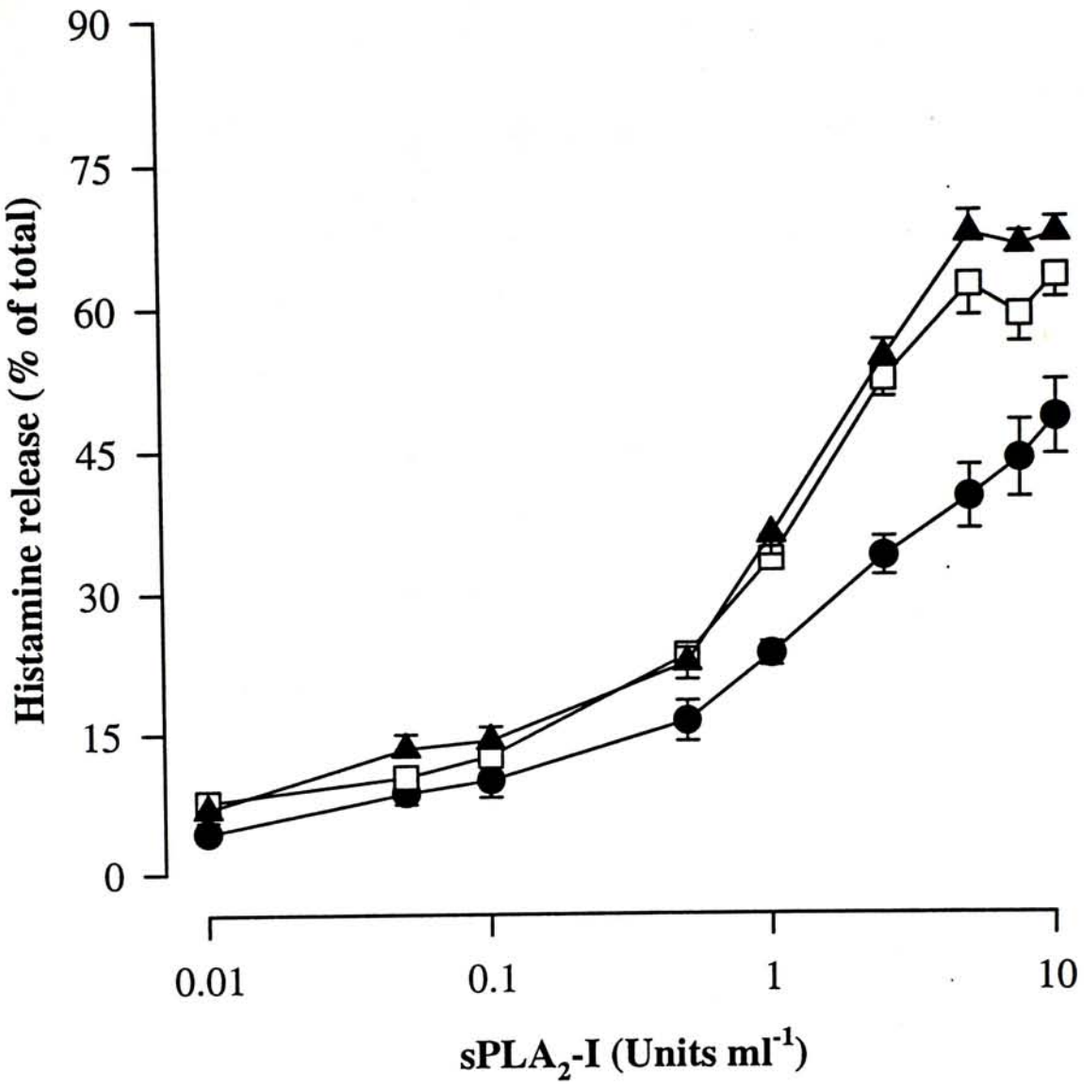

Figure 3.2 Effects of $sPLA_2$-I on the spontaneous histamine release from purified rat peritoneal mast cells. Cells were incubated with $sPLA_2$-I (0.01 to 10 U ml^{-1}) for 10 (●), 20 (□) and 30 min (▲). The spontaneous histamine release was 10.01 ± 0.81% (10 min), 13.32 ± 1.38% (20 min) and 16.18 ± 1.58% (30 min). Results are given as the means ± SEM for n = 5-10.

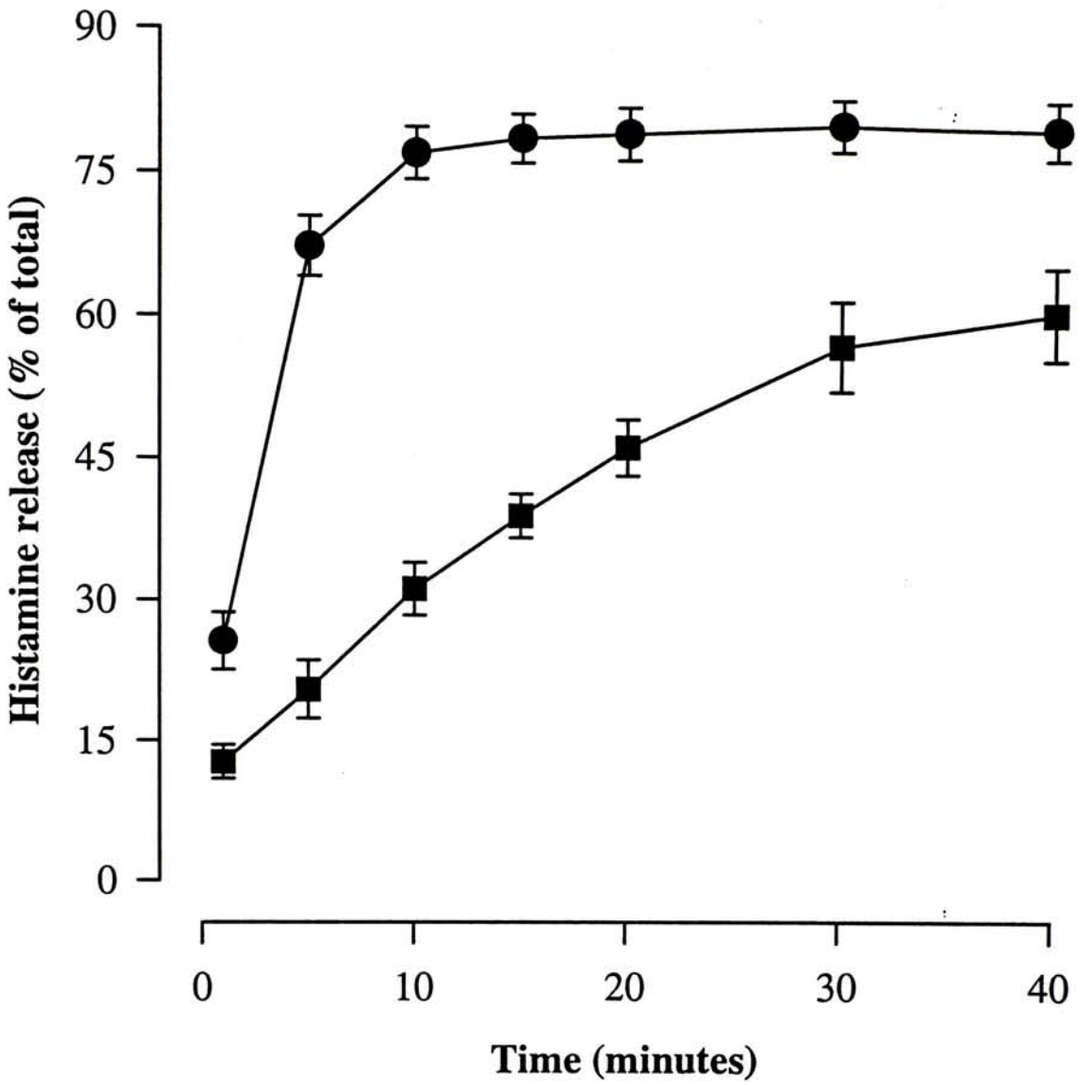

Figure 3.3 Time course of histamine release induced by $sPLA_2$-I (0.1 (■) and 1 (●) U ml^{-1}), from purified rat peritoneal mast cells. The spontaneous histamine release was 11.46 ± 1.16% for the 1 min incubation and 13.39 ±1.86% for the 40 min incubation. Results are given as the means ± SEM for n = 4.

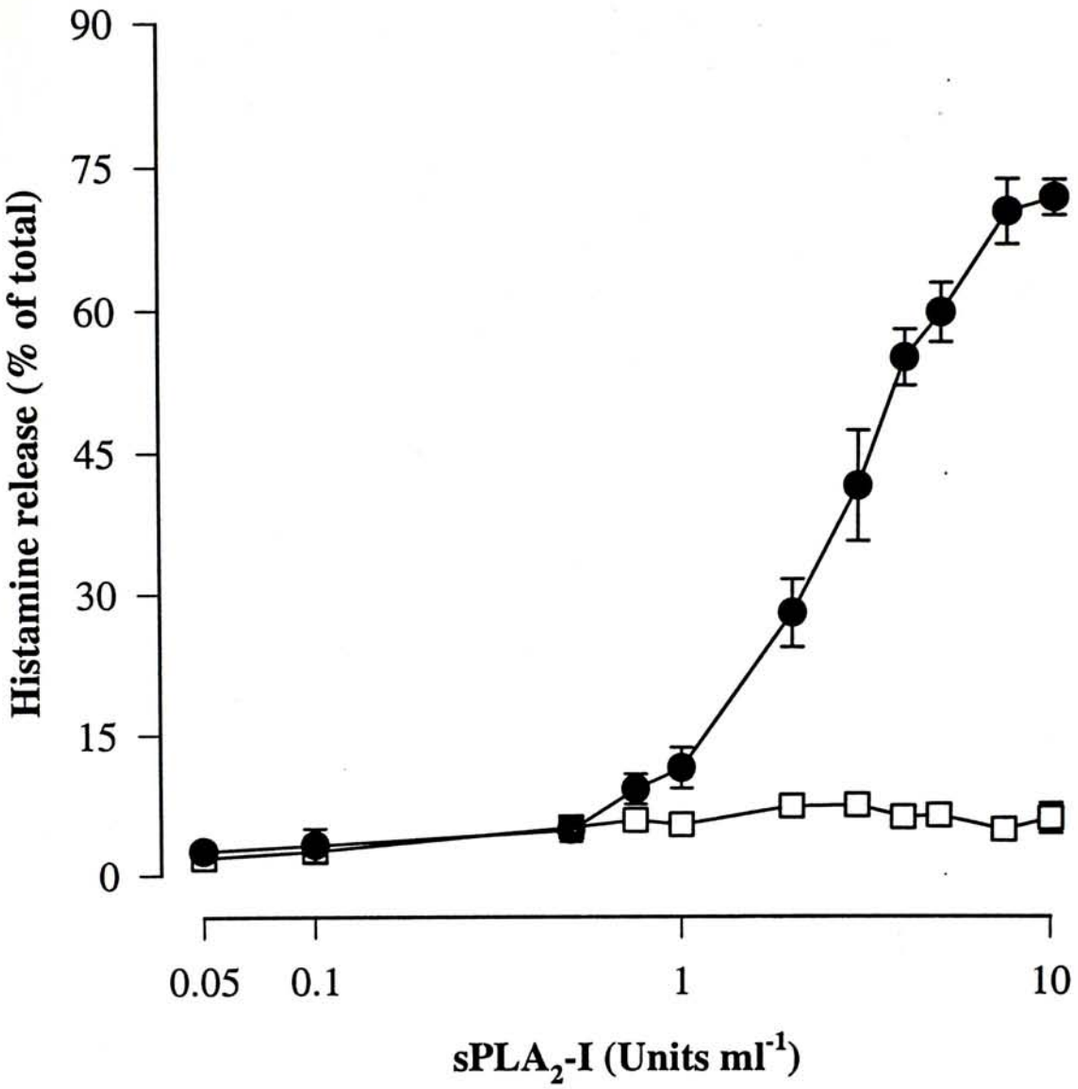

Figure 3.4 Effect of heat treated $sPLA_2$-I (100°C, 30 min) on the spontaneous histamine release from rat peritoneal mast cells. Cells were incubated with the heat treated enzyme (□) and the nonheat treated enzyme (●) for 10 min (0.05 to 10 U ml^{-1}). The spontaneous histamine release was 6.92 ± 1.20%. Results are given as the means ± SEM for n = 4-6.

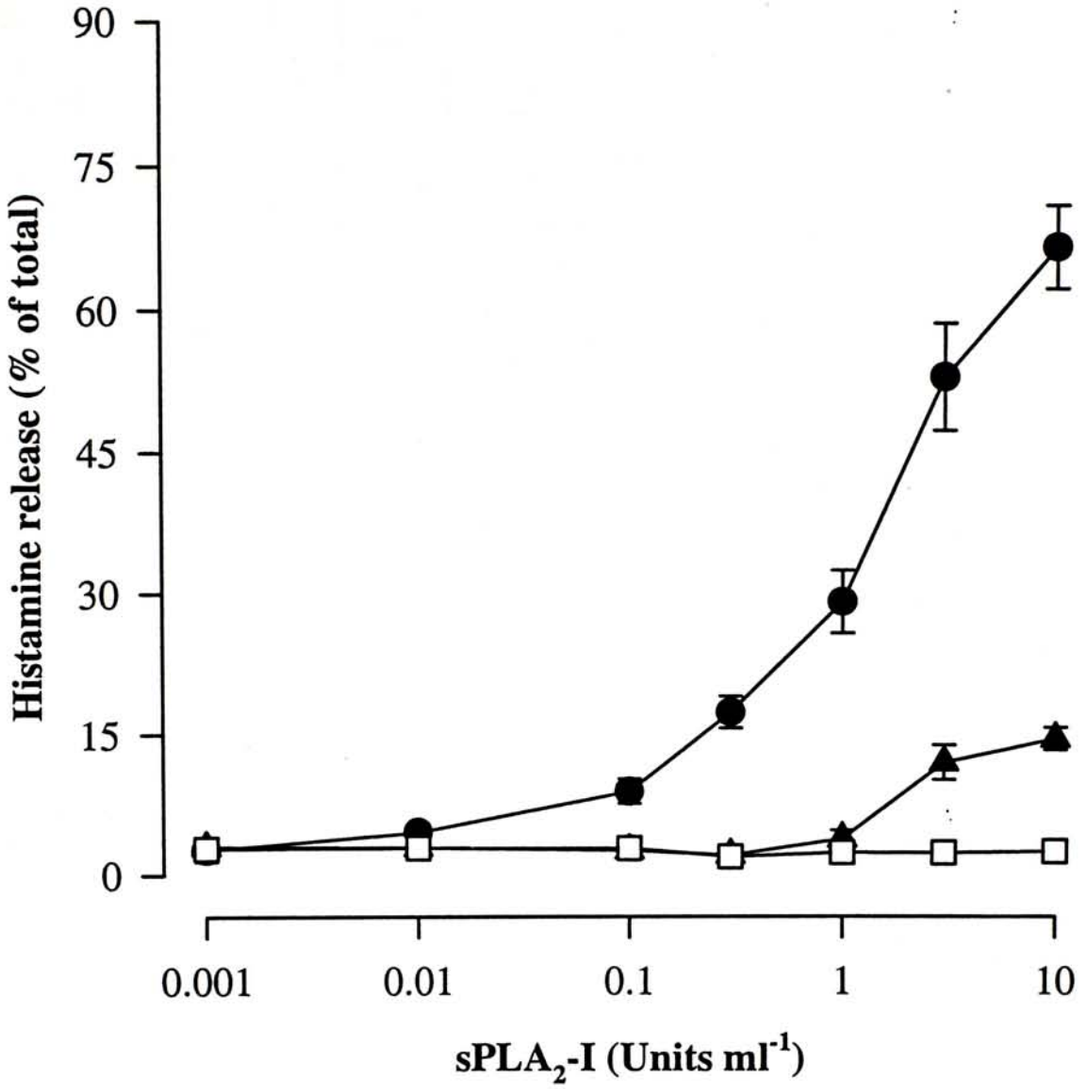

Figure 3.5 Effects of extracellular calcium on $sPLA_2$-I (0.001 to 10 U ml^{-1}) induced histamine release from purified rat peritoneal mast cells. Cells were preincubated for 5 min in Hepes buffer (●), CMF Hepes buffer (▲) and EDTA buffer (□) and then incubated with $sPLA_2$-I for 10 min. The spontaneous histamine release was 10.48 ± 1.40%. Results are given as the means ± SEM for n = 4-6.

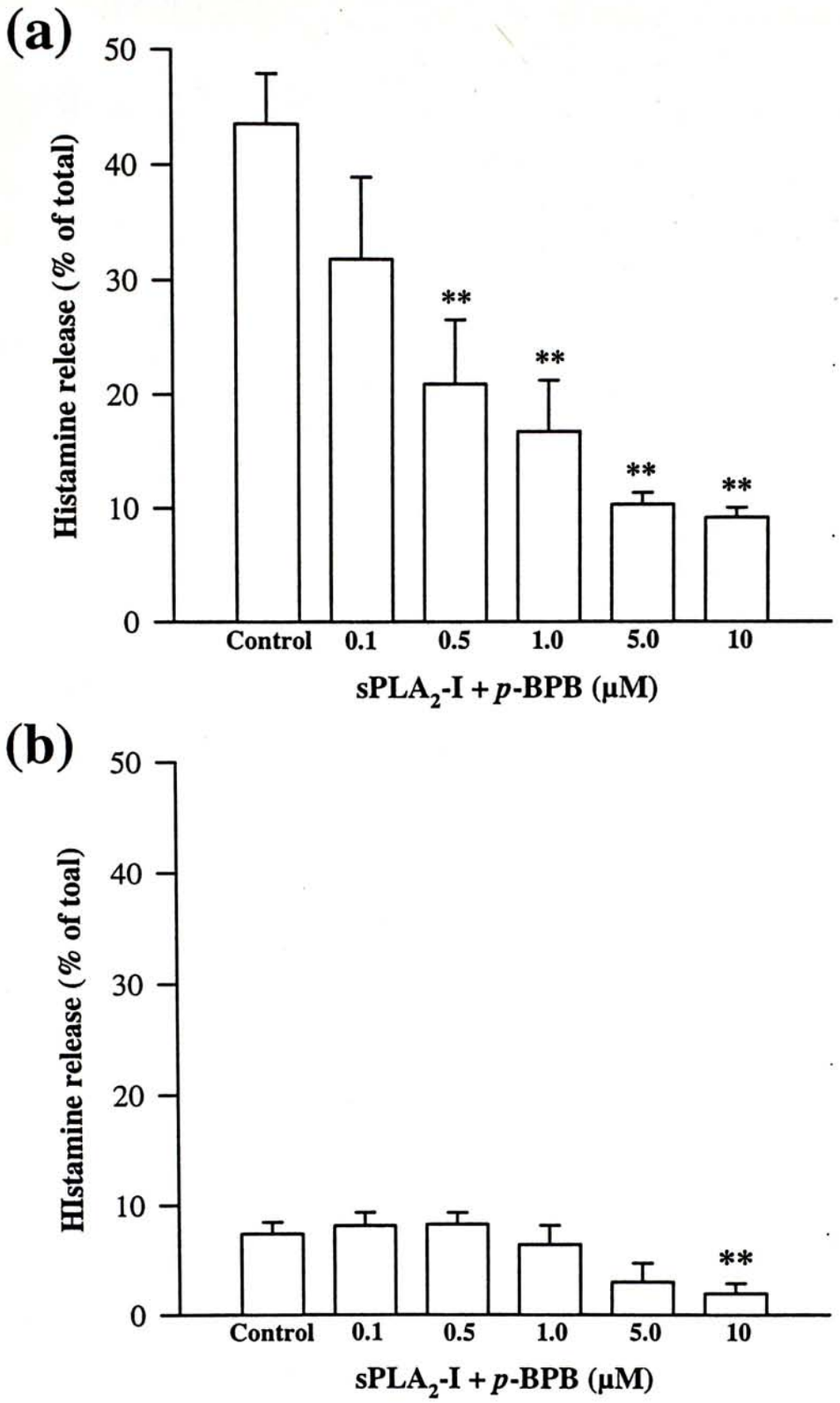

Figure 3.6 (a + b) Effects of *p*-BPB on $sPLA_2$-I induced histamine release from rat peritoneal mast cells. $sPLA_2$-I (1 U ml^{-1} (a) and 0.01 U ml^{-1} (b)) was incubated with *p*-BPB for 30 min at 37°C before the addition of cells. ** = $p \leq 0.01$ when compared with control. The spontaneous histamine release was 10.56 $\pm$ 0.38%. Values are given as the means $\pm$ SEM for n = 3-4.

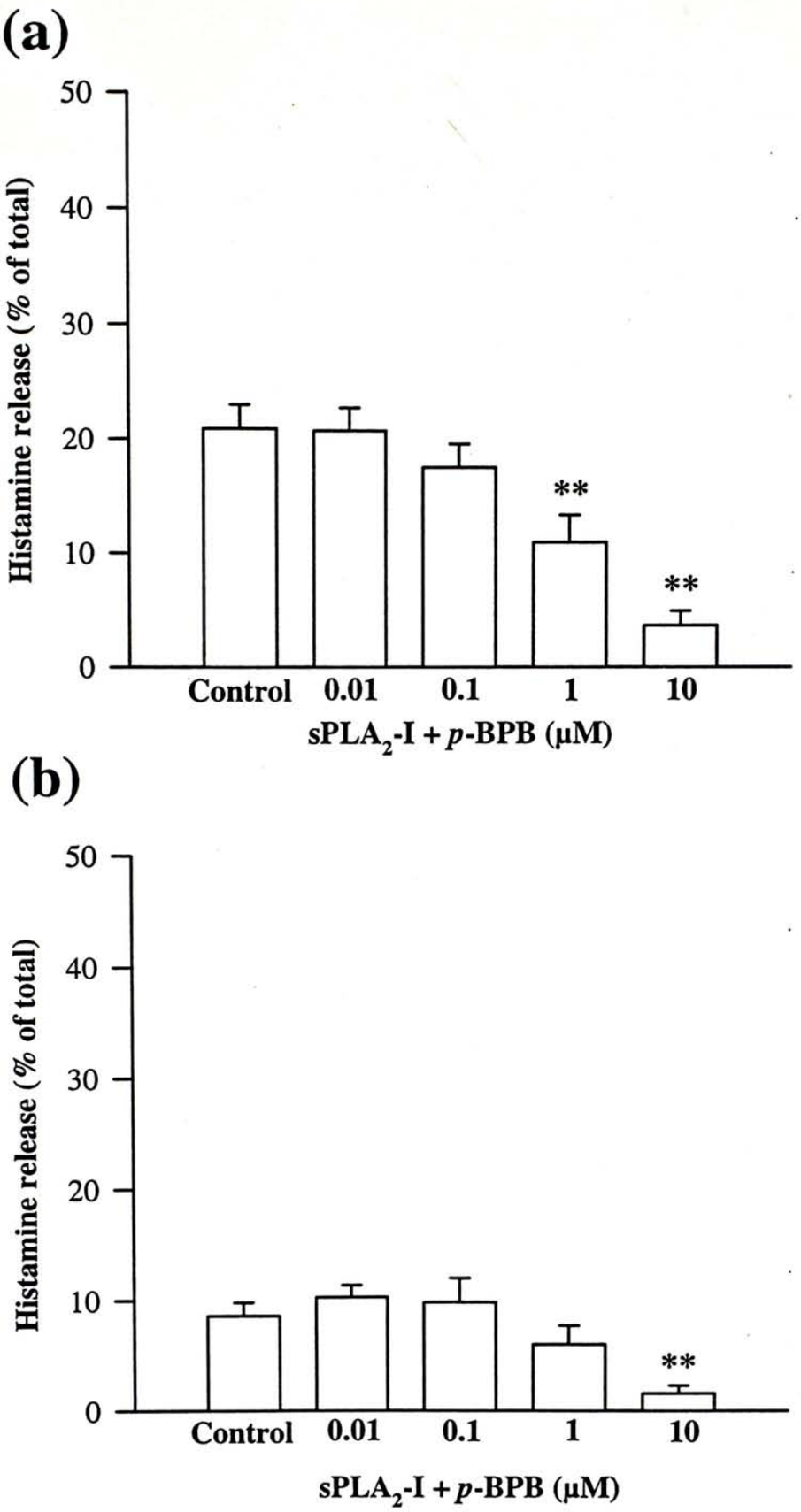

Figure 3.7 (a + b) Effects of *p*-BPB on $sPLA_2$-I induced histamine release from purified rat peritoneal mast cells. $sPLA_2$-I (0.1 U ml^{-1} (a) and 0.01 U ml^{-1} (b)) was incubated with *p*-BPB for 30 min at 37°C before the addition of cells. ** = $p \leq 0.01$ when compared with control. The spontaneous histamine release was 10.21 ± 0.85%. Results are given as the means ± SEM for n = 4.

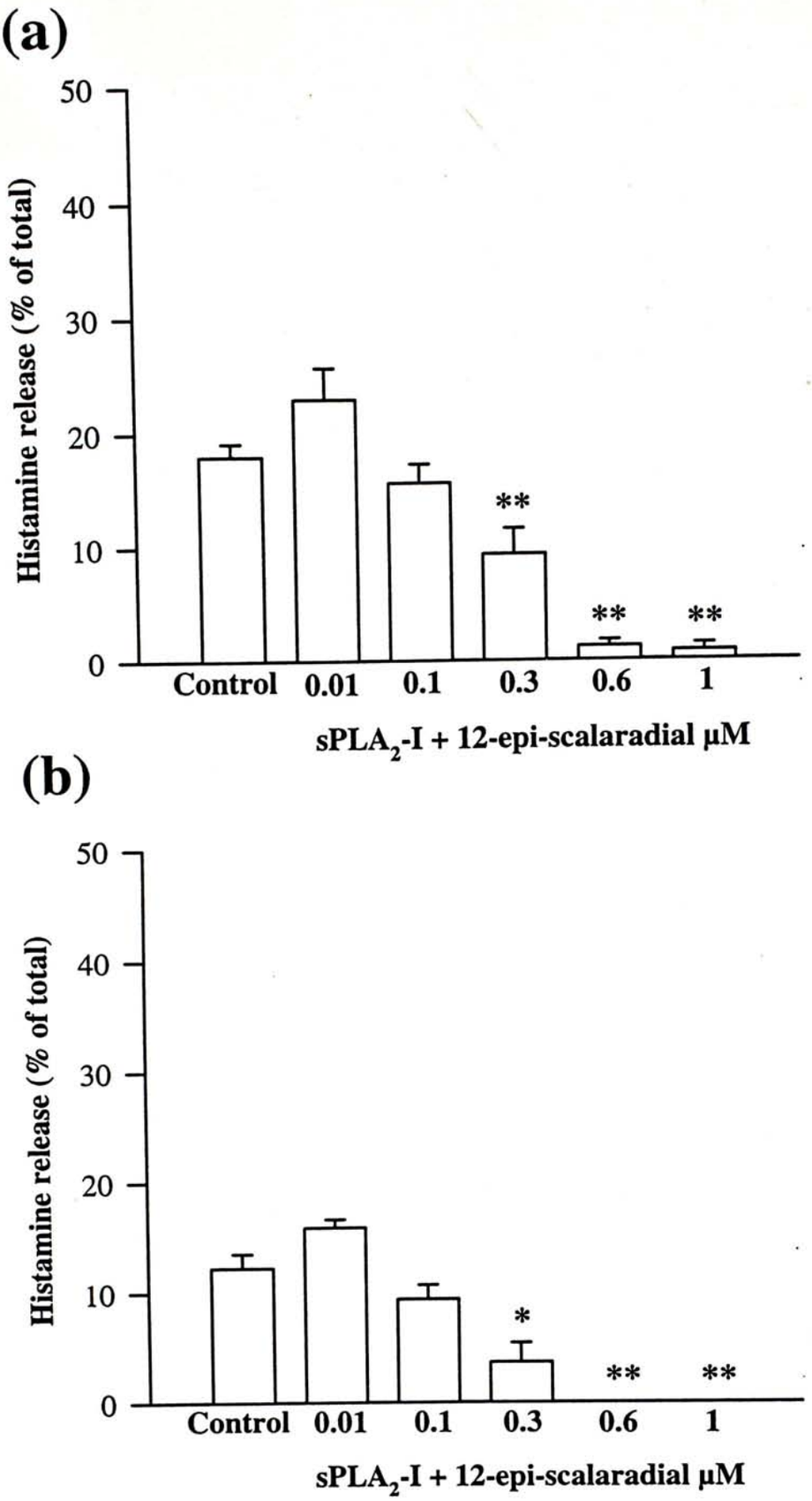

Figure 3.8 (a + b) Effects of 12-epi-scalaradial on $sPLA_2$-I induced histamine release from purified rat peritoneal mast cells. $sPLA_2$-I (0.1 U ml^{-1} (a) and 0.01 U ml^{-1} (b)) was incubated with 12-epi-scalaradial for 30 min at 37°C before the addition of cells. ** = $p \leq 0.01$ and * = ≤ 0.05 when compared with control. The spontaneous histamine release was 12.66 ± 0.63%. Results are given as the means ± SEM for n = 3-7.

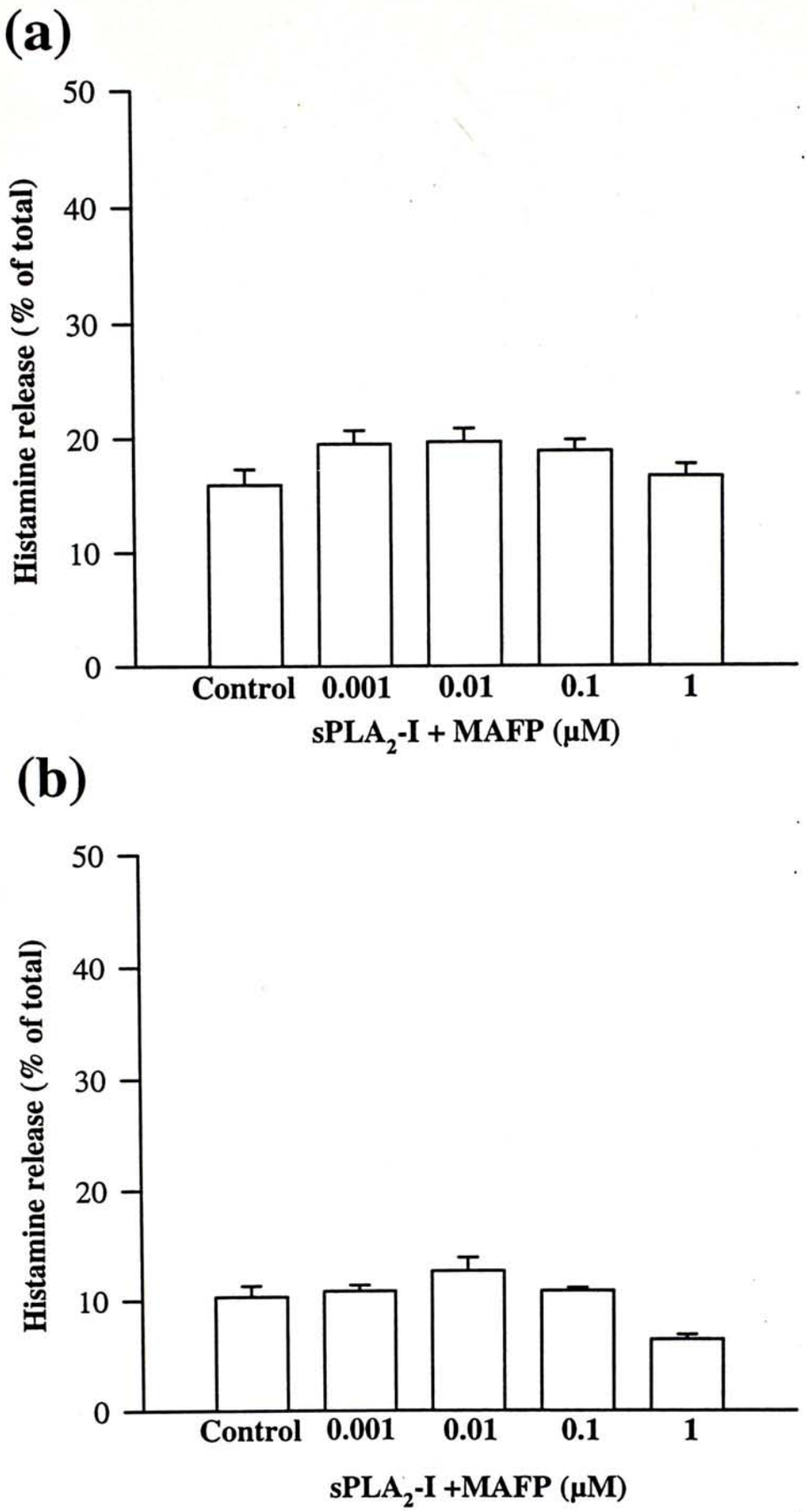

Figure 3.9 (a + b) Effects of MAFP on $sPLA_2$-I induced histamine release from purified rat peritoneal mast cells. $sPLA_2$-I (0.1 U ml^{-1} (a) and 0.01 U ml^{1} (b)) was incubated with MAFP for 30 min at 37°C before the addition of cells. The spontaneous histamine release was 11.33 ± 0.79%. Results are given as the means ± SEM for n = 5.

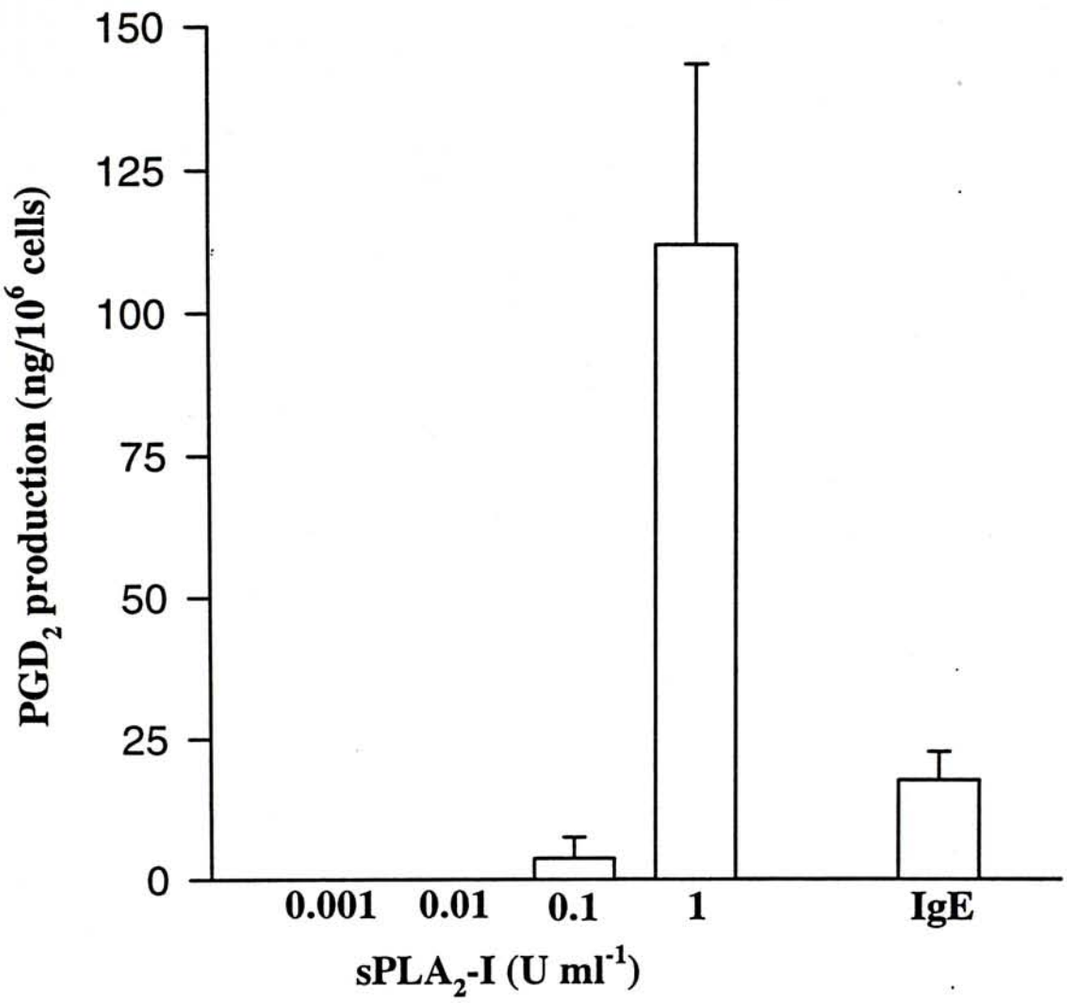

Figure 3.10 Effects of $sPLA_2$-I on PGD_2 production from purified rat peritoneal mast cells. Cells were incubated with $sPLA_2$-I for 20 min. As a positive control for PGD_2 production, cells were stimulated with anti-rat IgE for 10 min. Values are means ± SEM for n = 3.

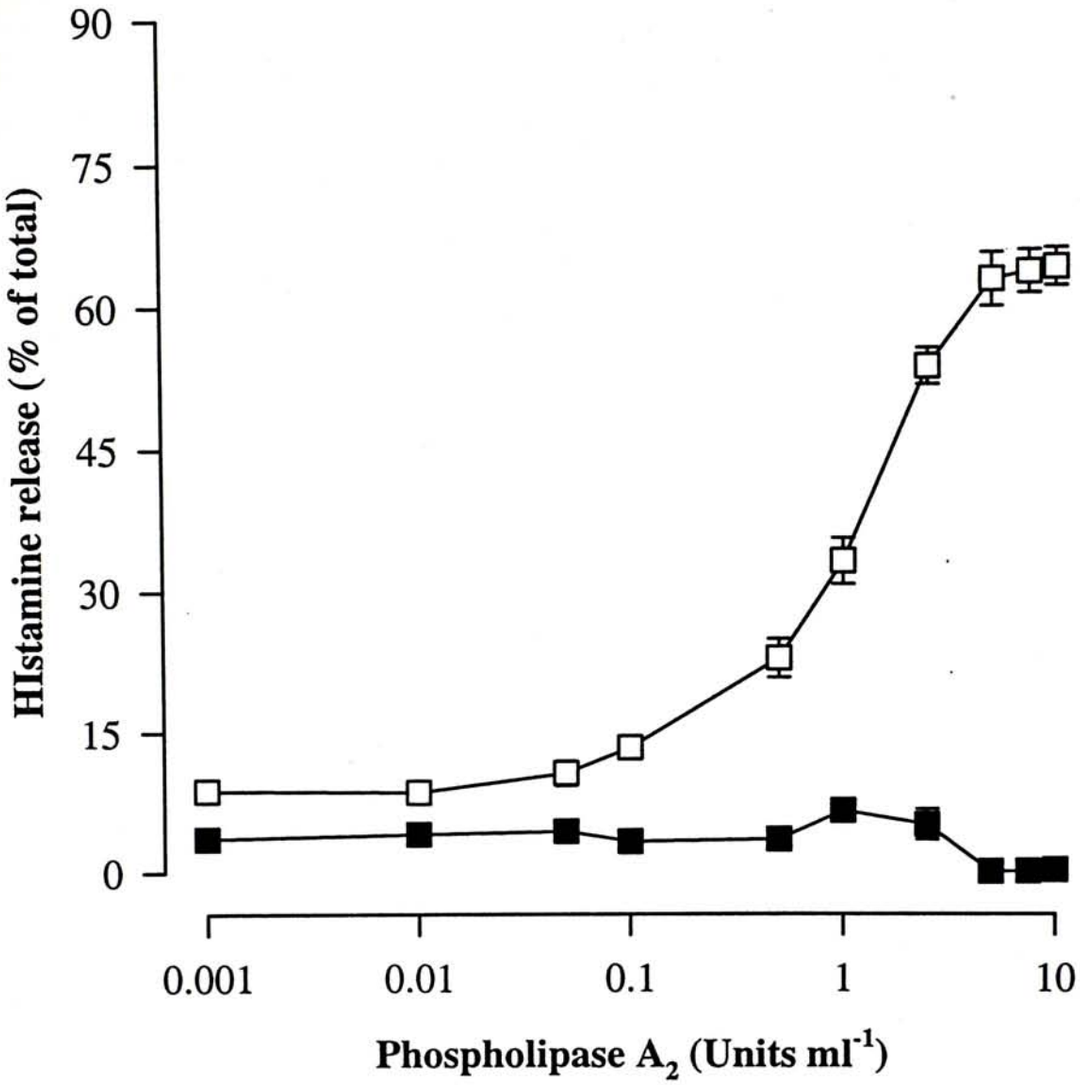

Figure 3.11 Effects of $sPLA_2$-I and $sPLA_2$-II on the spontaneous histamine release from purified rat peritoneal mast cells. Cells were incubated with $sPLA_2$-I (□) and $sPLA_2$-II (■) for 20 min (0.001 to 10 U ml^{-1}). The spontaneous histamine release was 7.73 ± 0.48%. Results are given as the means ± SEM for n = 3-8.

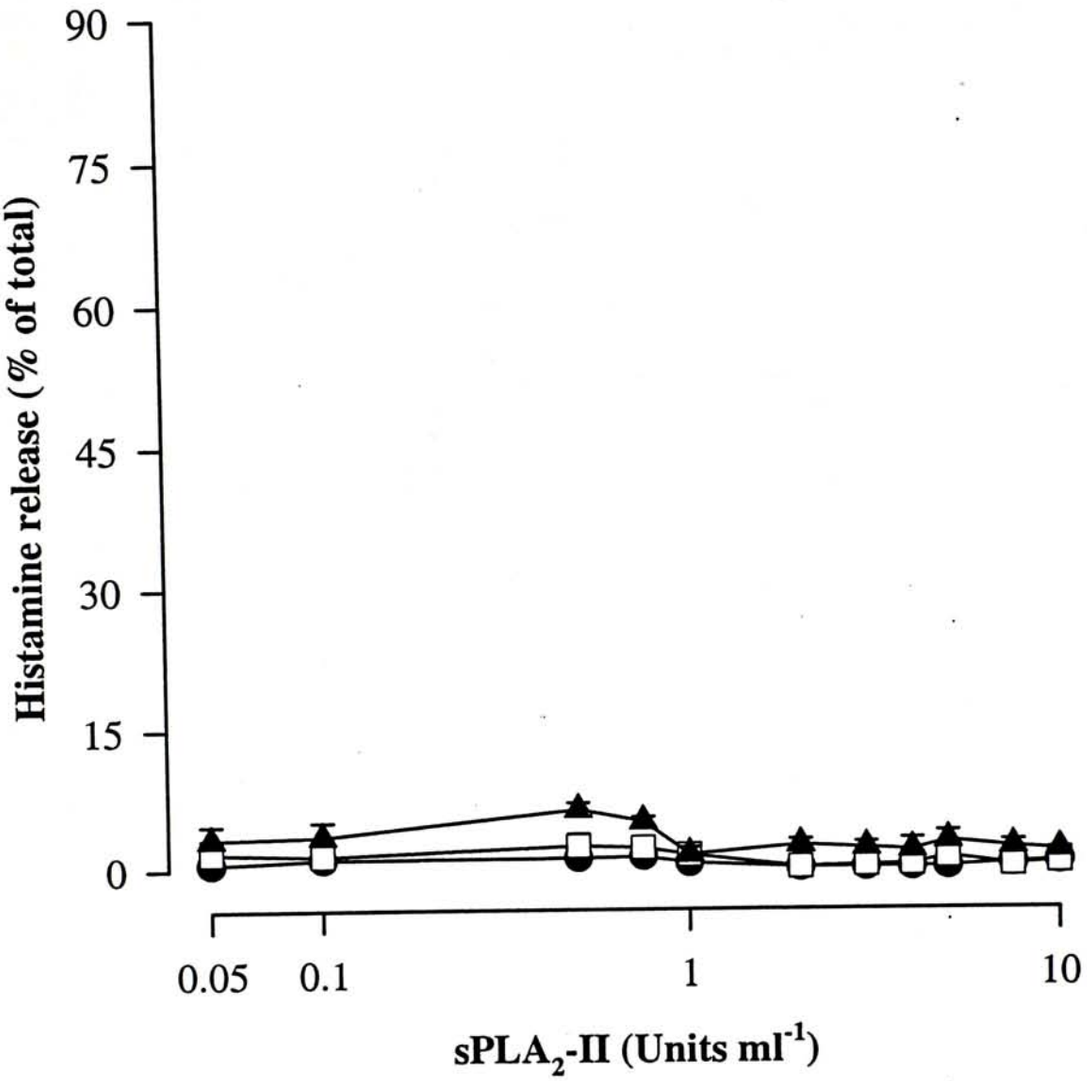

Figure 3.12 Effects of $sPLA_2$-II on the spontaneous histamine release from rat peritoneal mast cells. Cells were incubated with $sPLA_2$-II (0.05 to 10 U ml^{-1}) for 10 (●), 20 (□) and 30 min (▲). The spontaneous histamine release was 7.31 ± 1.15% (10 min), 8.67 ± 0.73% (20 min) and 9.08 ± 0.80% (30 min). Results are given as the means ± SEM for n = 5-6.

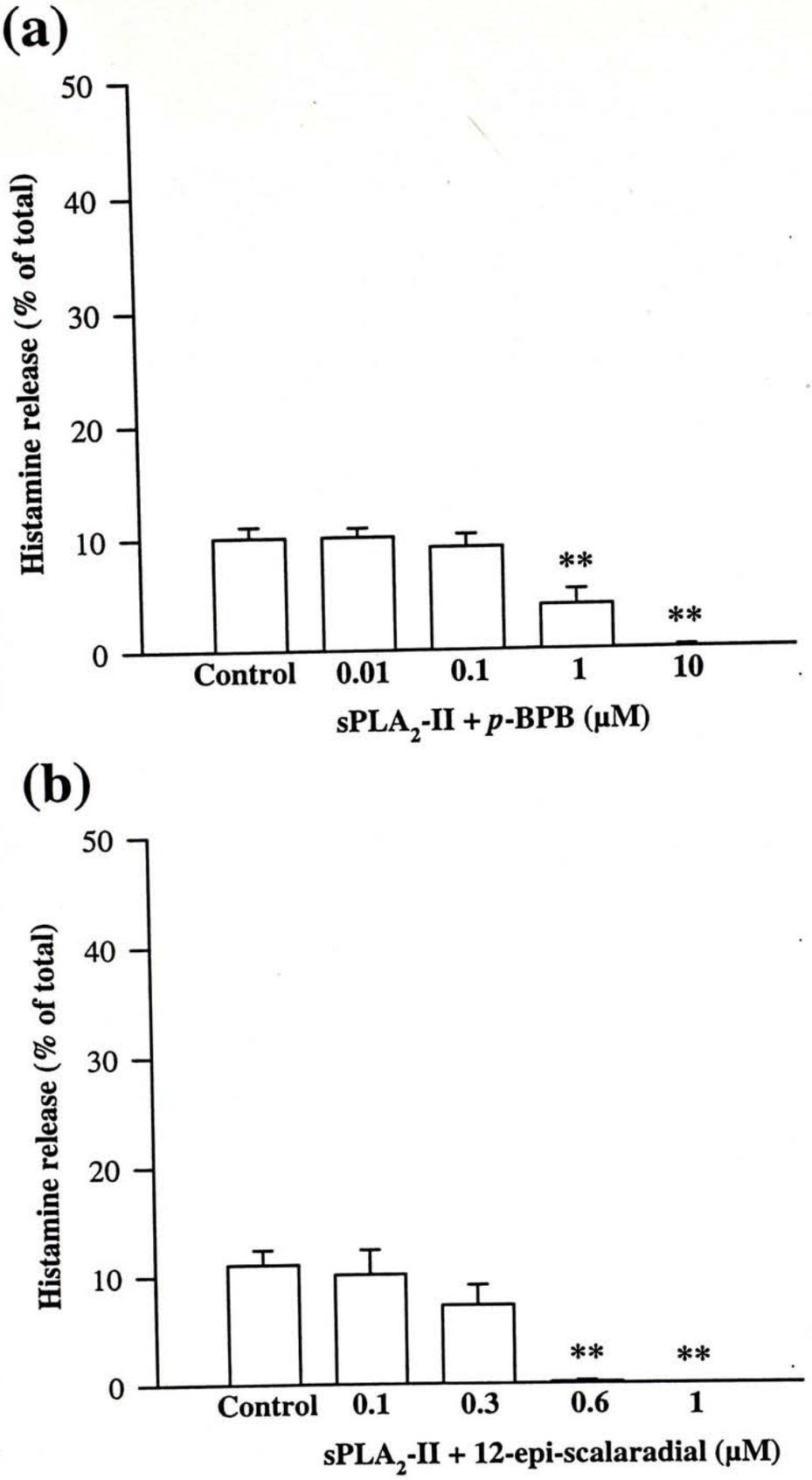

Figure 3.13 (a + b) Effects of *p*-BPB (a) and 12-epi-scalaradial (b) on $sPLA_2$-II induced histamine release from purified rat peritoneal mast cells. $sPLA_2$-II (1 U ml^{-1}) was incubated with *p*-BPB or 12-epi-scalaradial for 30 min at 37°C before the addition of cells. The spontaneous histamine release was 11.37 ± 0.63%. ** = $p \leq 0.01$ when compared with control. Results are given as the means ± SEM for n = 5-6.

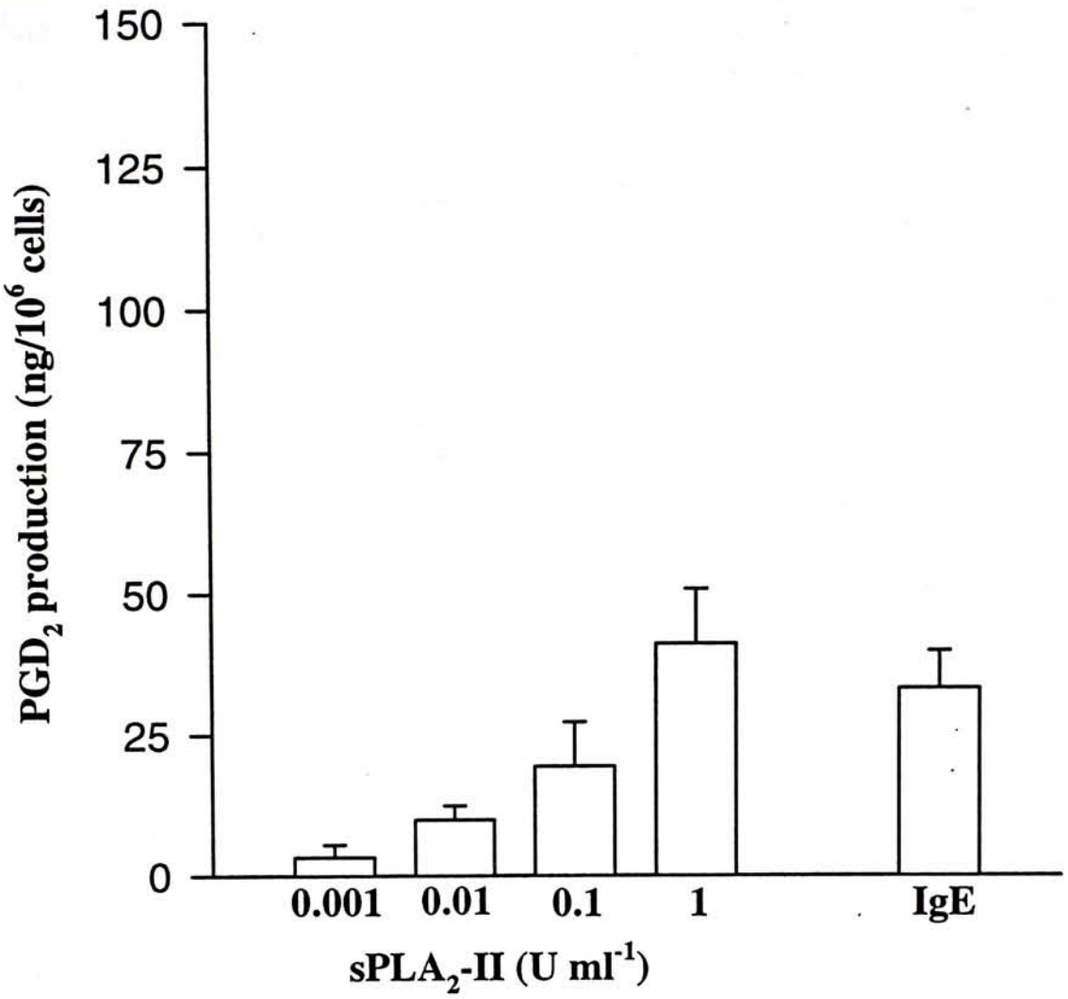

Figure 3.14 Effects of $sPLA_2$-II on PGD_2 production from purified rat peritoneal mast cells. Cells were incubated with $sPLA_2$-II for 20 min. As a positive control for PGD_2 production, cells were stimulated with anti-rat IgE for 10 min. Values are means ± SEM for n = 4-5.

Table 3.1 Effects of glucose and antimycin A on the histamine release induced by $sPLA_2$-I from rat peritoneal mast cells. Cells were preincubated in the various media for 20 min before the addition of $sPLA_2$-I or Compound 48/80 (48/80). Values are means ± SEM for n = 3-4. ** = $p \leq 0.01$ as compared to the appropriate control.

Histamine release (% of total)

	Control	**Glucose free**	**Antimycin A**
$sPLA_2$-I			
10 Units ml^{-1}	73.0 ± 1.4	70.9 ± 1.7	15.0 ± 2.2**
5.0 Units ml^{-1}	72.2 ± 1.3	67.7 ± 2.4	14.4 ± 1.7**
2.5 Units ml^{-1}	58.3 ± 6.3	57.4 ± 6.8	14.7 ± 1.3**
1.0 Unit ml^{-1}	41.8 ± 6.6	35.0 ± 7.7	14.5 ± 1.6**
48/80			
1.0 μg ml^{-1}	61.3 ± 2.0	60.6 ± 1.6	5.8 ± 4.3**
0.25 μg ml^{-1}	20.4 ± 3.5	20.6 ± 3.0	1.6 ± 1.0*

Table 3.2 Effects of *p*-BPB, mepacrine and aristolochic acid on $sPLA_2$-I induced histamine release from rat peritoneal mast cells. $sPLA_2$-I (1 U ml^{-1}) was pretreated with 10 μM of *p*-BPB, mepacrine and aristolochic acid for 5, 15, 30 and 60 min at 37°C. Cells were incubated for 20 min with inhibitor pretreated $sPLA_2$-I. $sPLA_2$-I induced histamine release was 45.96 ± 3.79%. The spontaneous histamine release was 9.59 ± 1.07%. ** = $p \leq 0.01$ and * = $p \leq 0.05$ as compared with control (45.96 ± 3.79%). Values are means ± SEM for n = 4-5.

Histamine release (% of total)

Time	*p*-BPB	Mepacrine	Aristolochic acid
5 min	17.34 ± 1.15**	34.29 ± 3.55*	42.87 ± 3.47
15 min	16.04 ± 1.23**	32.94 ± 3.90*	38.39 ± 4.57**
30 min	15.91 ± 2.12**	31.57 ± 2.80**	35.41 ± 2.52**
60 min	14.35 ± 1.88**	33.24 ± 3.47*	32.10 ± 3.12**

3.4 Discussion

3.4.1 Effects of $sPLA_2$-I on RPMC

The type I $sPLA_2$ enzyme isolated from *Naja naja* venom caused the release of histamine from rat peritoneal mast cells dose dependently. These results are in agreement with previous observations where the $sPLA_2$ enzyme isolated from *Naja naja* venom caused histamine release from rat peritoneal mast cells in a dose related manner (Choi *et al.*, 1989; Nagai *et al.*, 1991; Moreno *et al.*, 1992; Lloret & Moreno, 1993). Chi *et al.* (1982) also reported that the porcine pancreas PLA_2 caused the rapid release of histamine from rat peritoneal mast cells. Histamine release occurred within 5 min and plateaued after 10 min (2 U ml^{-1}). The histamine release observed here with 1 U ml^{-1} was also rapid within the first 5 min. The lower concentration of 0.1 U ml^{-1} released histamine slowly and plateaued after 30 min. This is consistent with Choi *et al.* (1989) who also observed a slow release of histamine within 15 minutes. However the enzyme concentration they tested was 100 fold greater.

In the present study, the removal of calcium from the extracellular medium reduced the histamine releasing activity of $sPLA_2$-I. The requirement for extracellular calcium here suggests that the enzymatic activity of $sPLA_2$-I may be involved in causing histamine release. Millimolar concentrations of external Ca^{2+} are required for the optimal enzymatic activity of $sPLA_2$ (Dennis, 1983). $sPLA_2$-I may exert its effect by hydrolysing the mast cell phospholipid plasma membrane and generate a lipid metabolite which may be involved in the process of histamine secretion. Alternatively,

$sPLA_2$-I may alter the mast cell plasma membrane structure through phospholipid hydrolysis. This could lead to an increased permeability to extracellular calcium ions. This influx of extracellular Ca^{2+} would lead to mast cell degranulation as Ca^{2+} plays an important role in the process of histamine secretion following cell activation (Pearce, 1985).

$sPLA_2$-I lost at least 90% of its histamine releasing ability when heated for 30 min at 100°C, suggesting that the natural structure of the enzyme is required, either for its enzymatic activity, or for some specific interactions with the plasma membrane. However, these results appear to differ from Chi *et al.* (1982) who observed that the porcine pancreas $sPLA_2$ (2 U ml^{-1}) retained its mast cell degranulating activity when heated for 1 hour at 100°C. De Haas *et al.* (1968) also reported that about 45% of the lipolytic activity of porcine pancreas $sPLA_2$ was retained after heat treatment for 1 hour at 98°C. The differences observed here may be due to the different source of enzymes or differences in the assay conditions used.

The removal of glucose together with the inhibition of the oxidative phosphorylation pathway with antimycin A, significantly reduced the histamine releasing activity of $sPLA_2$-I. An intracellular supply of ATP, is required to maintain the cells in a phosphorylated state, as protein phosphorylation plays a role in the secretory process (Gomperts, 1991). The requirement for ATP here would suggest that $sPLA_2$-I required a metabolically active cell in order to mediate its histamine releasing effect and also suggested that $sPLA_2$-I was having a specifc effect on the mast cell plasma membrane.

$sPLA_2$-I may exert its effect by hydrolysing the mast cell phospholipid plasma membrane. To further investigate this, the effects of some PLA_2 inhibitors on $sPLA_2$-I induced histamine release were examined. Several types of compounds have been reported to have PLA_2 inhibitory activity (for review see Chang *et al.*, 1987b; Gelb *et al.*, 1994) and some of these inhibitors work by competing with the phospholipid substrate for binding to the active site of the enzyme. It was observed here that both inhibitors, *p*-BPB and 12-epi-scalaradial, reduced the histamine release induced by $sPLA_2$-I. Chi *et al.* (1982) also reported that the porcine pancreas PLA_2 induced histamine release from rat peritoneal mast cells was reduced following *p*-BPB treatment (100 μM). *p*-BPB inactivates PLA_2 by alkylation of the histidine residue found in the catalytic site (Volwerk *et al.*, 1974; Roberts *et al.*, 1977). *p*-BPB has been shown to inhibit the PLA_2 isolated from *Vipera russelli* with an IC_{50} of 9 μM (Vallee *et al.*, 1979). 12-epi-scalaradial is thought to inhibit PLA_2 by imine formation between its aldehyde group and lysine residues in the PLA_2 enzyme and has been shown to inhibit the bee venom PLA_2 with an IC_{50} of 0.2 μM (Potts *et al.*, 1992).

Mepacrine and aristolochic acid also reduced the histamine releasing activity of $sPLA_2$-I at a concentration of 10 μM. This is in agreement with Moreno *et al.* (1992), who also observed inhibition of histamine release induced by the $sPLA_2$ isolated from *Naja naja* venom, with mepacrine and aristolochic acid at concentrations of 5 and 50 μg/ml respectively. Mepacrine is thought to work by altering the natural phospholipid substrate structure. It has been reported that large amounts of these inhibitors are required to produce significant inhibition of PLA_2 activity (Chang *et al.*, 1987b). Mepacrine (conc. $>$ 0.2 mM) was reported to interact directly with the membrane

phospholipid structure of human platelets and erythrocytes (Dise *et al.*, 1982). Moreno (1993) also observed that aristolochic acid (1 mM) inhibited the 5-lipoxygenase enzyme involved in AA metabolism. In order to minimise any non specific membrane interactions, the highest inhibitor concentration employed in these studies was 10 μM. In the present study, both 12-epi-scalaradial and *p*-BPB were more potent than mepacrine or aristolochic acid in inhibiting $sPLA_2$-I induced histamine release.

The histamine releasing activity of $sPLA_2$-I was unaffected following treatment with the $cPLA_2$ inhibitor MAFP. MAFP has been reported to inhibit A23187 induced LTB_4 production in human neutrophils with an IC_{50} of 0.1 μM (Huang *et al.*, 1994). This suggested that the histamine release observed here was mediated by $sPLA_2$-I itself.

These observations with the various PLA_2 inhibitors, together with the requirement for ATP, extracellular Ca^{2+} and the native structure of the enzyme, would suggest that the enzymatic activity of $sPLA_2$-I was in some way responsible for the observed histamine release. As more selective/specific $sPLA_2$-I inhibitors become available the exact mechanism of action of $sPLA_2$-I can be further investigated.

Hydrolysis of the plasma membrane phospholipid, by $sPLA_2$-I may generate a lipid substance(s), such as a free fatty acid, a lysophospholipid or related metabolites, which may be responsible for the induced histamine release. This unknown lipid substance may promote fusion of the mast cell plasma membrane with the secretory granules during exocytosis. Lysophosphatidylcholine (lysoPC) has been reported to cause

membrane fusion, but only at high concentrations of 300 μg/ml (Poole *et al.*, 1970). However, recent findings have modified this observation since lysophospholipids were shown to inhibit membrane fusion (Chernomordik *et al.*, 1993). Further work is needed to clarify the role of lysophospholipids in membrane fusion.

In resting platelets the outer layer of the plasma membrane is predominately occupied with neutral phospholipid such as phosphatidylcholine (PC) and sphingomyelin. The inner layer contains the negatively charged phospholipid, phosphatidylethanolamine (PE) and phosphatidyserine (PS) (Bevers *et al.*, 1983). It is possible that this asymmetric lipid distribution may also exist in the plasma membrane of mast cells. $sPLA_2$-I enzymes tend to display a phospholipid substrate specificity for PC and PE (Verheij *et al.*, 1981). PC on the outer layer of the plasma membrane could be hydrolysed by $sPLA_2$-I to yield a lipid substance. This lipid substance could then facilitate fusion between the plasma membrane and the secretory granules during exocytosis. LysoPC has been reported to inhibit mouse mast cell degranulation (Chernomordik *et al.*, 1993). However, Martin & Lagunoff (1979) reported that the lysophospholipids, lysoPC, lysoPE and lysoPS all caused histamine release from rat peritoneal mast cells. Lysophospholipids are single chain amphiphiles and are capable of forming detergent micelles in aqueous media when suspended at concentrations above their critical micelle concentration (CMC). At and above the CMC, detergent micelles lyse cells. Concentrations above 10 μM of all three lysophospholipids were required to cause histamine release independent of the presence of the stimulating agent, concanavalin A (con A). However, lysoPS also caused con A dependent histamine release at concentrations below 10 μM. The biphasic dose response curve

obtained with lysoPS was the result of differential actions on the mast cell membrane above and below its CMC. Below the CMC lysoPS interacts with the mast cell membrane in a noncytotoxic manner and potentiated con A induced histamine release, whereas above the CMC histamine was released in a cytotoxic manner. Although all three lysophospholipids caused substantial con A independent histamine release, this release was mediated through a cytotoxic mechanism. These observations would suggest that lysoPC does not seem to be responsible for the effects observed in the present study, but some other unidentified lipid metabolite may be involved.

Alternatively, $sPLA_2$-I may mediate its effect by binding to a specific binding site on the mast cell surface and simply act as a signalling molecule. $sPLA_2$-I has been shown to regulate cell proliferation (Arita *et al.*, 1991; Hanasaki & Arita, 1992) and induce contraction in isolated guinea pig lung parenchyma strips (Sommers *et al.*, 1992; Kanemasa *et al.*, 1992). These physiological effects are thought to be mediated through $sPLA_2$-I receptor sites. $sPLA_2$-I receptors have been identified and cloned from a variety of tissues and cells (Lambeau *et al.*, 1994; Ishizaki *et al.*, 1994; Ancian *et al.*, 1995). These cloned receptors are homologous to the macrophage mannose receptor (Taylor *et al.*, 1990) and the recently cloned DEC-205 receptor in dendritic cells (Jiang *et al.*, 1995). One characteristic of these receptors is their ability to internalise upon ligand binding. Internalisation following ligand binding has been reported for a number of cell surface receptors such as epidermal growth factor (Gorden *et al.*, 1978), insulin (Goldfine, 1981) and IL-1 (Matsushima *et al.*, 1986). These internalised receptor-ligand complexes are then degraded in intracellular lysosomal compartments. Internalisation here could direct the action of $sPLA_2$-I into

the mast cell following receptor binding.

Binding of $sPLA_2$-I to a specific binding site may lead to an intracellular signalling event which eventually leads to histamine secretion. Kishino *et al.* (1995) reported that the porcine pancreatic $sPLA_2$ augmented PGE_2 production in rat mesangial cells. This effect was receptor mediated and did not require the enzymatic action of $sPLA_2$-I. They found that incubation of rat mesangial cells with an enzymatically inactive mutant G30S, induced PGE_2 synthesis with the same potency as the wild-type porcine $sPLA_2$-I. Other enzymatically inactive mutants without receptor binding affinity were without effect on PGE_2 synthesis. Xing *et al.* (1995) also reported that short term exposure of NIH 3T3 cells to the porcine pancreatic $sPLA_2$ did not generate any significant AA release from intact cells. However, AA generation was significantly increased following a 6 hour exposure. This effect was unaffected by *p*-BPB treatment. They proposed that this AA release was a receptor mediated event. In the present study prior to AA generation mast cell degranulation may be facilitated through a receptor mediated process.

Arita & Hanasaki (1993) reported that rat peritoneal mast cells as well as platelets and macrophages did not display any specific binding for the porcine pancreas $sPLA_2$. These observations suggested that mast cells do not have any binding site for the $sPLA_2$-I. However, this porcine pancreas $sPLA_2$ binding site did not recognise the snake venom $sPLA_2$ isolated from *Naja naja* venom (Ohara *et al.*, 1995). Further work is needed to determine if binding sites for the type I $sPLA_2$ isolated from *Naja naja* venom are present on the surface of the mast cell plasma membrane.

The type I $sPLA_2$ not only caused the release of histamine from rat peritoneal mast cells, but it also generated a significant amount of PGD_2. Fonteh *et al.* (1994) also reported that treatment of mouse mast cells (BMMC) with 1 μg/ml of the $sPLA_2$ isolated from *Naja naja* venom for 4 min resulted in the selective release of AA for PGD_2 and TXB_2 production. In this study, PGD_2 production was observed with an $sPLA_2$ concentration of 1 U ml^{-1} within 20 min. This PGD_2 could be produced by hydrolysis of the membrane phospholipid (PC) to yield free AA. This free AA on the outside of the cell, could be rapidly taken up by the cell itself or some other cells and would then be available to the enzymes involved in AA metabolism inside the cell.

Alternatively, if $sPLA_2$-I was acting as a signalling molecule and was activating an intracellular signal transduction pathway, then PGD_2 could be generated by activation of the intracellular $cPLA_2$. The high molecular weight $cPLA_2$ has been detected in rat and mouse mast cell lines (RBL-2H3 and BMMC) (Murakami *et al.*, 1992b; Nakatani *et al.*, 1994a & b). This $cPLA_2$ is thought to be responsible for liberating the AA involved in eicosanoid production following cell activation. In addition to an increase in the $[Ca^{2+}]_i$, which is involved in translocating the $cPLA_2$ to the cellular membrane, protein phosphorylation is also required for full activation of the $cPLA_2$ (Glover *et al.*, 1995; Hirasawa *et al.*, 1995; for review see Clark *et al.*, 1995). It has been reported that the $cPLA_2$ in mast cells becomes phosphorylated following IgE receptor crosslinkage (Nakatani *et al.*, 1994a; Currie *et al.*, 1994). Xing *et al.* (1995) reported that following a 6 hour exposure of NIH 3T3 cells to the porcine pancreatic $sPLA_2$, a significant amount of AA was produced. This AA release was receptor mediated.

In the present study, significant histamine release was observed with $sPLA_2$-I concentrations greater than 1 U ml^{-1} and PGD_2 production was only observed with an $sPLA_2$-I concentration of 1 U ml^{-1}. These effects were observed over a short time period of 20 min. The observations reported by Kishino *et al.* (1995) and Xing *et al.* (1995) required a long time span of 24 and 6 hours respectively and were receptor mediated. In the present study $sPLA_2$-I may mediate its effects through its catalytic site by hydrolysis of the mast cell plasma membrane phospholipid. This hydrolysis could alter the membrane structure and lead to an influx of extracellular ions such as calcium, which can induce histamine secretion. The free AA from this lipid hydrolysis may be quickly metabolised by the cyclo-oxygenase enzyme to produce PGD_2. Further work is needed to determine the exact mechanism(s) of action of $sPLA_2$-I on the surface of the mast cell plasma membrane.

3.4.2 Effects of $sPLA_2$-II on RPMC

While the type I $sPLA_2$ isolated from *Naja naja* venom caused the release of histamine from rat peritoneal mast cells, it was interesting that the type II $sPLA_2$ enzyme purified from *Crotalus altrox* venom did not seem to have any effect. Indeed the histamine release observed was generally not greater than 10%. Murakamai *et al.* (1993a) observed that rat peritoneal mast cells incubated with the type II PLA_2 purified from rat platelets (10 and 40 μg ml^{-1}) released histamine in a dose dependent manner. These concentrations were within the range examined in this study (0.4 to 40 μg ml^{-1}). Similarly, histamine release was also observed when rat peritoneal mast cells were incubated with the type II PLA_2 purified from *Crotalus durissus terrificus* (5 U ml^{-1})

(Lloret & Moreno, 1993). These differences maybe due to the different enzymes employed in the studies and methods of induction. LysoPS was added as a cofactor for histamine release and cells were isolated from Wistar rats in the studies by Murakamai *et al.* (1993). Lloret & Moreno (1993) used BSA as a cofactor for histamine release. In the present study lysoPS and BSA were not employed in the histamine release reaction. These differences in the experimental procedure may explain the different observations reported.

Although cells usually released only about 10% histamine when incubated with the type II $sPLA_2$, this release was reduced following pretreatment with the PLA_2 inhibitors, *p*-BPB and 12-epi-scalaradial. This would suggest that the catalytic site of the enzyme may be involved in causing the low histamine release observed here. The type II $sPLA_2$ enzymes do display a phospholipid substrate specificity for PE and PS (Kudo *et al.*, 1993). These phospholipid substrates are usually found in the inner layer of the plasma membrane of resting platelets (Bevers *et al.*, 1983). This may explain why there was no significant release of histamine observed here with $sPLA_2$-II, if its effect is mediated through the hydrolysis of membrane phospholipid.

While $sPLA_2$-II did not seem to produce any significant histamine release, PGD_2 was produced in a dose dependent manner. Murakami *et al.* (1991a & 1993a), observed that incubation of rat peritoneal mast cells with the type II $sPLA_2$ (0 to 20 μg/ml) purified from rat platelets did not generate any significant amount of PGD_2. The differences observed here, may again be due to the different experimental procedures employed in the various studies, as was discussed with the histamine results. The

observations here, would suggest that $sPLA_2$-II may bind to a binding site on the mast cell plasma membrane, act as a signalling molecule and lead to the generation of PGD_2. However no such $sPLA_2$-II receptors have been identified on mast cells.

It is possible that $sPLA_2$-II may bind to the $sPLA_2$-I receptor site with low affinity, Although Ca^{2+} is not required for receptor binding, the Ca^{2+}binding loop of the $sPLA_2$ enzyme which is essential for catalytic activity, is involved in interaction with the receptor site (Lambeau *et al.*, 1995). The residues Gly-30 and Asp-49 which are conserved in all of the $sPLA_2$ enzymes sequenced so far (Davidson & Dennis, 1990), are involved in the binding step (Lambeau *et al.*, 1995). In addition, Leu-31 is essential for receptor binding of the porcine pancreatic $sPLA_2$ (Lambeau *et al.*, 1995). Several different residues are found in position 31 in the other $sPLA_2$ sequences (Davidson & Dennis, 1990) and this may determine the binding affinity of the enzyme. Lambeau *et al.* (1994) reported that the cloned $sPLA_2$-I receptor, like the native receptor in skeletal muscle membranes, binds the group II secreted PLA_2, from platelets and synovial fluid with a K_d value of 0.8 ± 0.2 nM where K_d represents the equilibrium binding constant. This receptor also binds the group I porcine pancreas $sPLA_2$ with a higher K_d of 10 ± 3 nM. Binding of $sPLA_2$-II to such a site may lead to the activation of a signalling pathway or, upon binding $sPLA_2$-II may undergo internalization. This could direct the action of $sPLA_2$-II into the target cell. Lipid hydrolysis of the plasma membrane phospholipid could lead to a transient release and increase in free AA for the production of PGD_2. Further work is needed to determine if binding sites for the group II $sPLA_2$ exist on the surface of the mast cell plasma membrane.

Bevers *et al.* (1983) reported that in activated platelets there appears to be a translocation of the inner phospholipid PE and PS to the outer layer of the plasma membrane. It is possible that in activated mast cells such a lipid translocation could also occur. This would make the phospholipid substrates PS and PE accessible to the $sPLA_2$-II. The effect of the $sPLA_2$ enzymes on stimulated mast cells was next investigated (Part 2).

PART 2: Effects of $sPLA_2$ on stimulated rat peritoneal mast cells

3.5 Results

3.5.1 Effects of $sPLA_2$-I on histamine release from immunologically activated RPMC

Preliminary experiments with a mixed population of rat peritoneal mast cells were carried out to see if the $sPLA_2$-I enzyme would have any effects on immunologically activated cells. Anti-rat IgE was used as the immunological stimulus at a 1/1,000 dilution of the supplied stock. Cells were preincubated with $sPLA_2$-I for 10 min subsequent to challenge with anti-rat IgE. Histamine release was then allowed to proceed for a further 10 min. Non releasing concentrations of $sPLA_2$-I between 0.05 and 0.5 U ml^{-1}, significantly enhanced the anti-rat IgE induced histamine release dose dependently (Fig 3.15). As illustrated in table 3.3 the experimentally observed histamine release induced by anti-rat IgE from $sPLA_2$-I pretreated cells was far above the sum of the individual effects. Differences between the observed and additive effects were compared using paired Student's *t* tests. No significant enhancement was observed at concentrations higher than 0.5 U ml^{-1} as $sPLA_2$-I itself was causing significant histamine release.

Experiments were repeated with purified rat peritoneal mast cells. Similarly cells were preincubated with $sPLA_2$-I for 10 min before challenge with anti-IgE (1/100) for a further 10 min. Once again, concentrations of $sPLA_2$-I between 0.005 and 1 U ml^{-1},

significantly enhanced the anti-IgE induced histamine release dose dependently (Fig 3.16 and table 3.4). Concentrations greater than 1 U ml^{-1} caused significant histamine release alone, so enhancement was not observed. When purified cells were challenged with a non-releasing concentration of anti-IgE (1/10,000), a significant enhancement was again observed in a dose dependent manner (Fig 3.17 and table 3.5).

3.5.2 Effects of preincubation time on $sPLA_2$-I enhanced histamine release from immunologically activated RPMC

With an $sPLA_2$-I concentration of 0.1 U ml^{-1}, a significant enhancement was observed with cells preincubated with the enzyme for 10 min, when compared with 1 and 5 min ($p \leq 0.05$). The longer activation time (10 min) with anti-IgE also produced a more significant enhancement of the anti-IgE induced histamine release (Fig 3.18a + b).

3.5.3 Effects of *p*-BPB on $sPLA_2$-I enhanced histamine release from immunologically activated RPMC

In order to characterise the mechanism of the enhanced anti-IgE induced histamine release, the effects of *p*-BPB pretreatment on $sPLA_2$-I were investigated. It was observed that *p*-BPB itself reduced the anti-rat IgE induced histamine release (Table 3.6). Cells were incubated with *p*-BPB for 10 min and subsequently challenged with anti-rat IgE for a further 10 min. These cells released less histamine than the corresponding controls. *p*-BPB reduced the histamine release induced both by $sPLA_2$-I and anti-IgE. However a reduction of the enhanced histamine release was only

observed with *p*-BPB concentrations of 1 and 10 μM with 0.1 U ml^{-1} of $sPLA_2$-I as illustrated in table 3.7a. Enhancement was determined by comparing the experimentally observed histamine release with the sum of the individual effects in the presence of *p*-BPB. The enhanced IgE induced histamine release was not reduced with the lower concentrations of *p*-BPB tested (0.01 and 0.1 μM). With the lower $sPLA_2$-I concentration of 0.01 U ml^{-1}, a significant reduction was again observed with 1 and 10 μM of *p*-BPB (Table 3.7b).

3.5.4 Effects of 12-epi-scalaradial on $sPLA_2$-I enhanced histamine release from immunologically activated RPMC

12-epi-scalaradial was similar to *p*-BPB in that the histamine release induced by anti-rat IgE was significantly reduced (Table 3.6). Table 3.8a & b shows the effect of 12-epi-scalaradial on the enhancing activity of $sPLA_2$-I. The $sPLA_2$-I (0.1 U ml^{-1}) enhanced histamine release was significantly reduced with 0.6 and 1 μM of 12-epi-scalaradial. Similar reductions were also observed with 0.01 U ml^{-1} of $sPLA_2$-I.

3.5.5 Effects of MAFP on $sPLA_2$-I enhanced histamine release from immunologically activated RPMC

Fig 3.19a & b shows that the $sPLA_2$-I enhanced histamine release was unaffected by pretreatment with the $cPLA_2$ inhibitor MAFP. Table 3.6 also shows that MAFP had no effect on anti-IgE induced histamine release from purified rat peritoneal mast cells.

3.5.6 Effects of $sPLA_2$-I on PGD_2 production from immunologically activated RPMC

Cells were preincubated for 10 min with $sPLA_2$-I and subsequently challenged with anti-rat IgE (10 min). Both histamine and PGD_2 measurements were made. Fig 3.20a and table 3.9a show that histamine release was significantly enhanced with 0.1 and 1 U ml^{-1} of $sPLA_2$-I. However this enhancement was not observed with the corresponding PGD_2 produced (Fig 3.20b and table 3.9b). Although a significant enhancement of PGD_2 production was observed with an $sPLA_2$-I concentration of 0.01 U ml^{-1}, it should be noted here that the number of observations is three and more experiments are needed to clarify this observation.

3.5.7 Effects of flurbiprofen and zileuton on $sPLA_2$-I induced histamine release and enhanced histamine release from RPMC

Fig 3.21a & b shows that the cyclo-oxygenase inhibitor flurbiprofen had no inhibitory effect on $sPLA_2$-I induced histamine release. Flurbiprofen was also without effect on the enhanced histamine release induced by anti-IgE (Fig 3.22a & b). The effects of zileuton, a reversible inhibitor of 5-lipoxygenase, were also examined (Carter *et al.*, 1991). An IC_{50} value of 0.8 μM was reported by Bell *et al.* (1993) in blocking ionophore induced LTB_4 production from purified rat peritoneal mast cells. Zileuton was without effect on $sPLA_2$-I induced histamine release (Fig 3.23a). Zileuton also had no inhibitory effect on the enhancing activity of $sPLA_2$-I (Fig 3.23b).

3.5.8 Effects of $sPLA_2$-II on histamine release from immunologically activated RPMC

Although $sPLA_2$-II did not have any significant effect on the spontaneous histamine release, a significant enhancement of histamine release was observed in the presence of anti-IgE (Fig 3.24 and table 3.10). Concentrations of $sPLA_2$-II between 0.005 and 5 U ml^{-1} showed a synergistic effect on the anti-rat IgE induced histamine release. However at the highest concentration of 10 U ml^{-1}, the enhancing activity was diminished. Indeed the histamine released in the presence of $sPLA_2$-II was insignificantly different from that induced by anti-IgE alone. These preliminary experiments were performed with a mixed population of rat peritoneal mast cells.

A similar enhancement was again observed with purified cells (Fig 3.25 and table 3.11). This synergistic effect was observed at all $sPLA_2$-II concentrations examined. However with an $sPLA_2$-II concentration of 10 U ml^{-1}, the enhanced histamine release was lower than that observed with 1 U ml^{-1}. The histamine release observed was 52.96 $\pm$ 3.68% (10 U ml^{-1}) compared with 63.38 $\pm$ 4.03% at a concentration of 1 U ml^{-1}. Similarly when a non releasing concentration of anti-IgE (1/10,000) was used to activate $sPLA_2$-II preincubated cells, this synergistic effect was again observed (Fig 3.26 and table 3.12). This enhanced histamine release was one fold lower with 10 U ml^{-1} when compared to 1 U ml^{-1} of $sPLA_2$-II.

3.5.9 Effects of preincubation time on $sPLA_2$-II enhanced histamine release from immunologically activated RPMC

With an $sPLA_2$-II concentration of 0.1 U ml^{-1}, enhancement of anti-IgE induced release was observed at all preincubation times tested. There was no difference observed with the two activation times with anti-IgE (Fig 3.27a & b).

3.5.10 Effects of *p*-BPB on $sPLA_2$-II enhanced histamine release from immunologically activated RPMC

Although *p*-BPB reduced the histamine release induced by anti-IgE and $sPLA_2$-II, a reduction of the enhanced histamine release was observed at *p*-BPB concentrations of 1 and 10 μM with 1 U ml^{-1} of $sPLA_2$-II (Table 3.13).

3.5.11 Effects of 12-epi-scalaradial on $sPLA_2$-II enhanced histamine release from immunologically activated RPMC

Table 3.14 shows the effect of 12-epi-scalaradial on the enhancing activity of $sPLA_2$-II. The $sPLA_2$-II (1 U ml^{-1}) enhanced histamine release was significantly reduced with 0.6 and 1 μM of 12-epi-scalaradial.

3.5.12 Effects of MAFP on $sPLA_2$-II enhanced histamine release from immunologically activated RPMC

Fig 3.28 shows that the $sPLA_2$-II enhanced histamine release was unaffected by pretreatment with the $cPLA_2$ inhibitor MAFP.

3.5.13 Effects of $sPLA_2$-II on PGD_2 production from immunologically activated RPMC

Fig 3.29a & b shows the effect of $sPLA_2$-II preincubated cells activated by anti-rat IgE on PGD_2 production and histamine release. Although anti-rat IgE induced histamine release was significantly enhanced dose dependently, PGD_2 production was only significantly enhanced at 0.1 and 1 U ml^{-1} of $sPLA_2$-II (Table 3.15a & b).

3.5.14 Effects of flurbiprofen and zileuton on $sPLA_2$-II induced histamine release and enhanced histamine release from RPMC

Fig 3.30a & b shows that flurbiprofen had no inhibitory effect on $sPLA_2$-II induced histamine release and was also without effect on the enhanced histamine release from purified rat peritoneal mast cells. Zileuton was also without effect on $sPLA_2$-II induced histamine release and the enhanced histamine release induced by anti-IgE (Fig 3.31a & b).

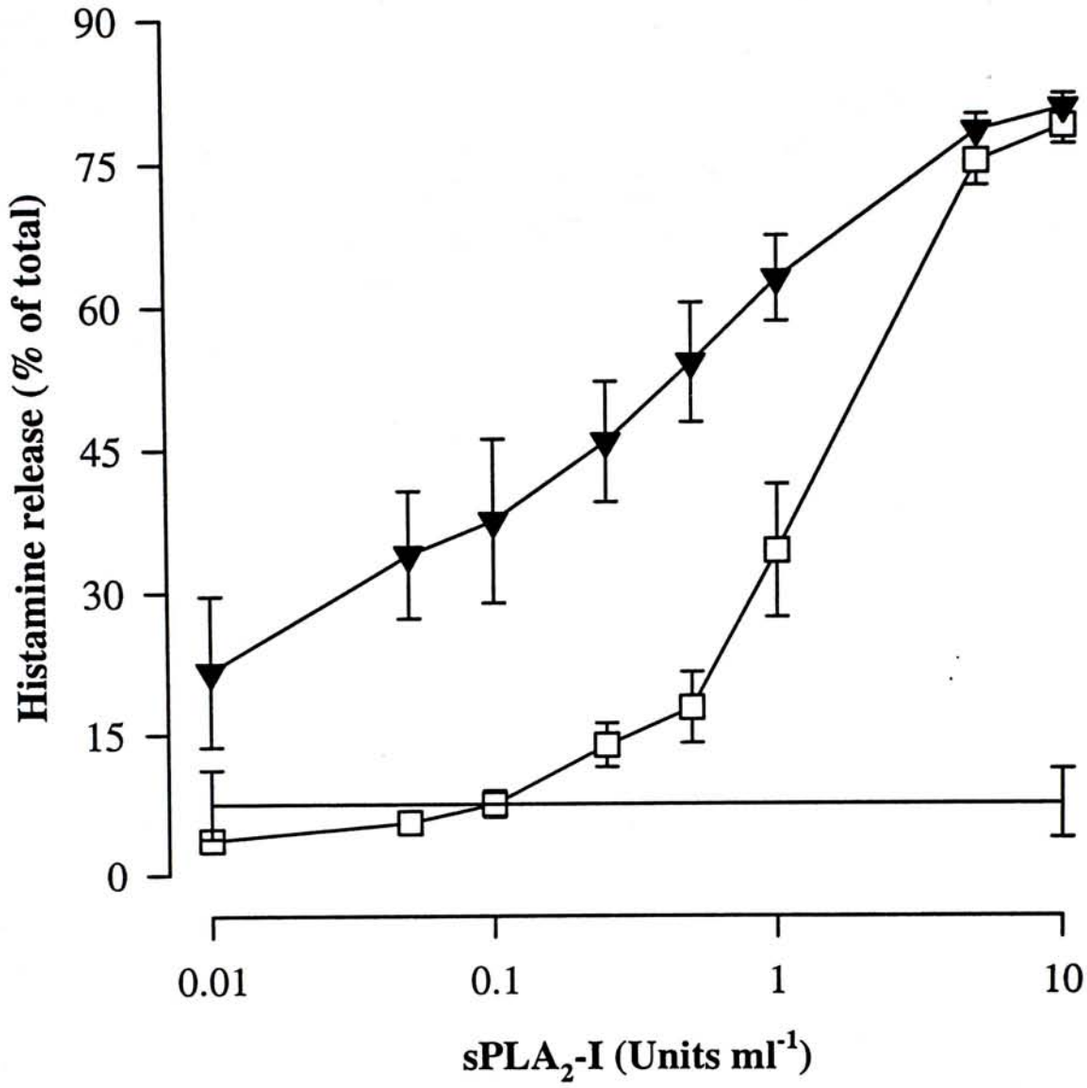

Figure 3.15 Effects of $sPLA_2$-I on anti-rat IgE induced histamine secretion from rat peritoneal mast cells. Control cells were incubated with $sPLA_2$-I (0.01 to 10 U ml^{-1}) for 20 min (□). Anti-rat IgE stimulated cells were pretreated with $sPLA_2$-I for 10 min and stimulated for a further 10 min with anti-rat IgE (▼). Anti-rat IgE (1/1,000) induced histamine release was 7.48 ± 3.66% (—). The spontaneous histamine release was 5.89 ± 0.52%. Results are given as the means ± SEM for n = 3-5.

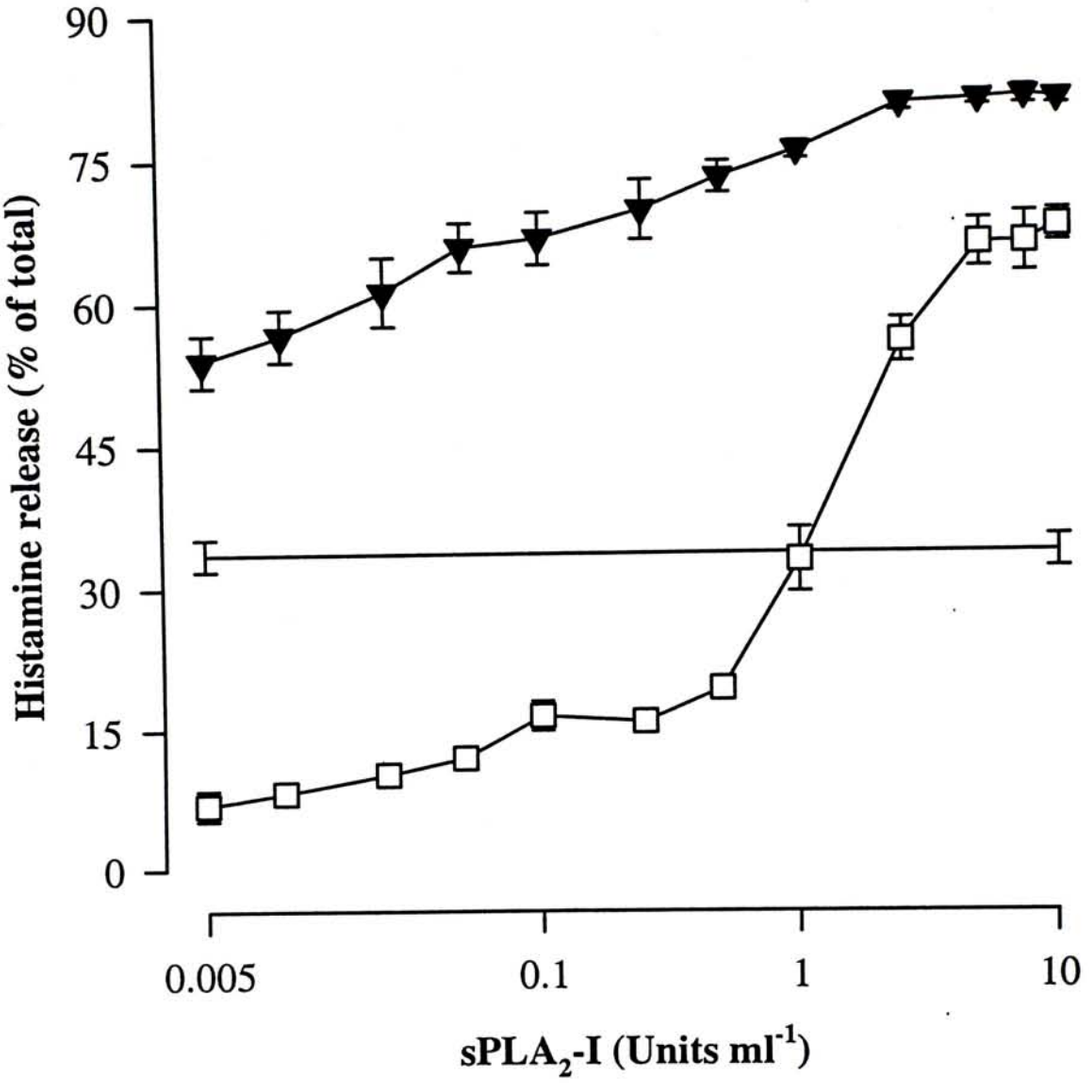

Figure 3.16 Effects of $sPLA_2$-I on anti-rat IgE induced histamine secretion from purified rat peritoneal mast cells. Control cells were incubated with $sPLA_2$-I (0.005 to 10 U ml^{-1}) for 20 min (□). Anti-rat IgE stimulated cells were pretreated with $sPLA_2$-I for 10 min and stimulated for a further 10 min (▼). Anti-rat IgE (1/100) induced histamine release was 33.57 ± 1.70% (—). The spontaneous histamine release was 8.73 ± 0.91%. Results are given as the means ± SEM for n = 5-6.

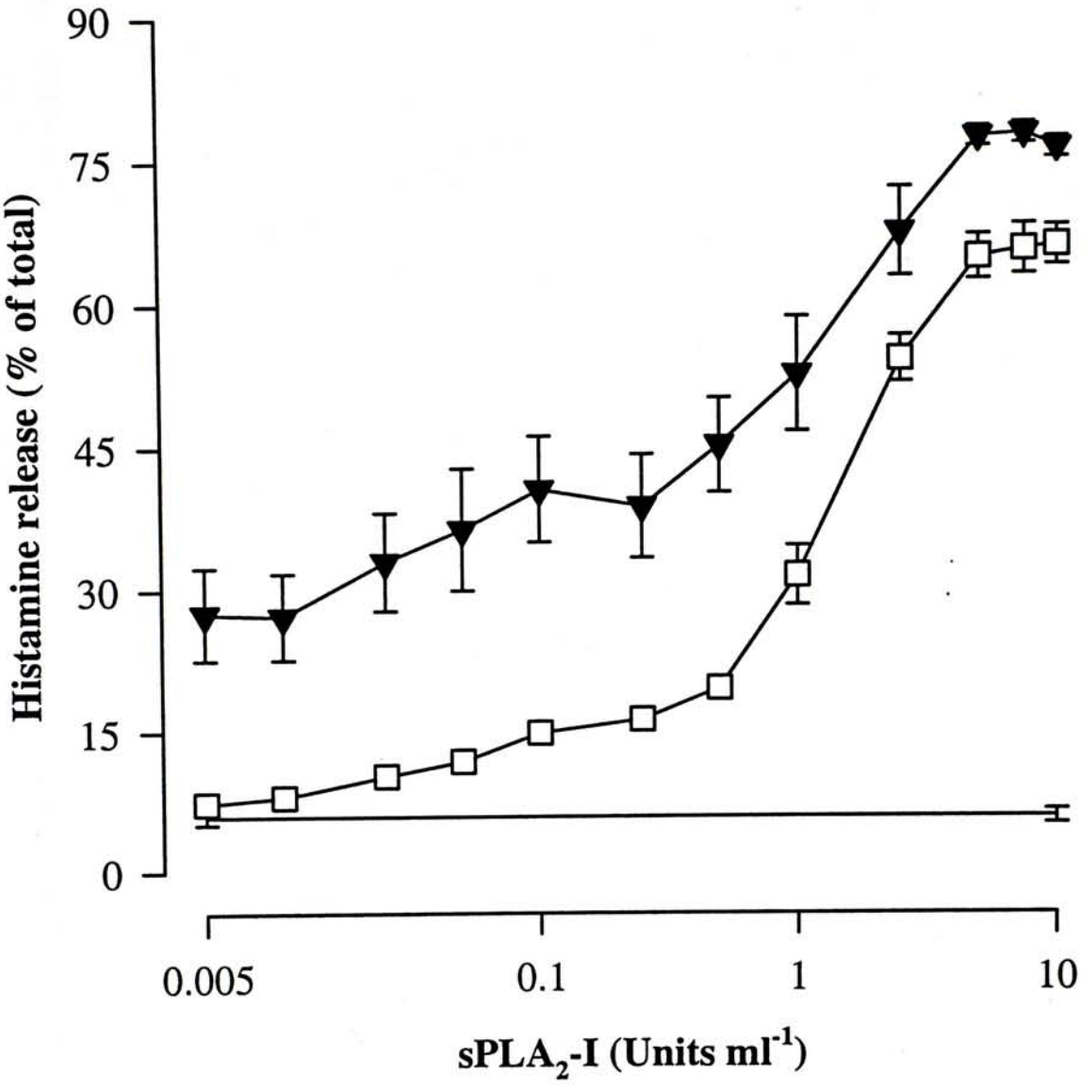

Figure 3.17 Effects of $sPLA_2$-I on anti-rat IgE induced histamine secretion from purified rat peritoneal mast cells. Control cells were incubated with $sPLA_2$-I (0.005 to 10 U ml^{-1}) for 20 min (□). Anti-rat IgE stimulated cells were pretreated with $sPLA_2$-I for 10 min and stimulated for a further 10 min (▼). Anti-rat IgE (1/10,000) induced histamine release was 5.85 ± 0.76% (—). The spontaneous histamine release was 8.73 ± 0.91%. Results are given as the means ± SEM for n = 4-7.

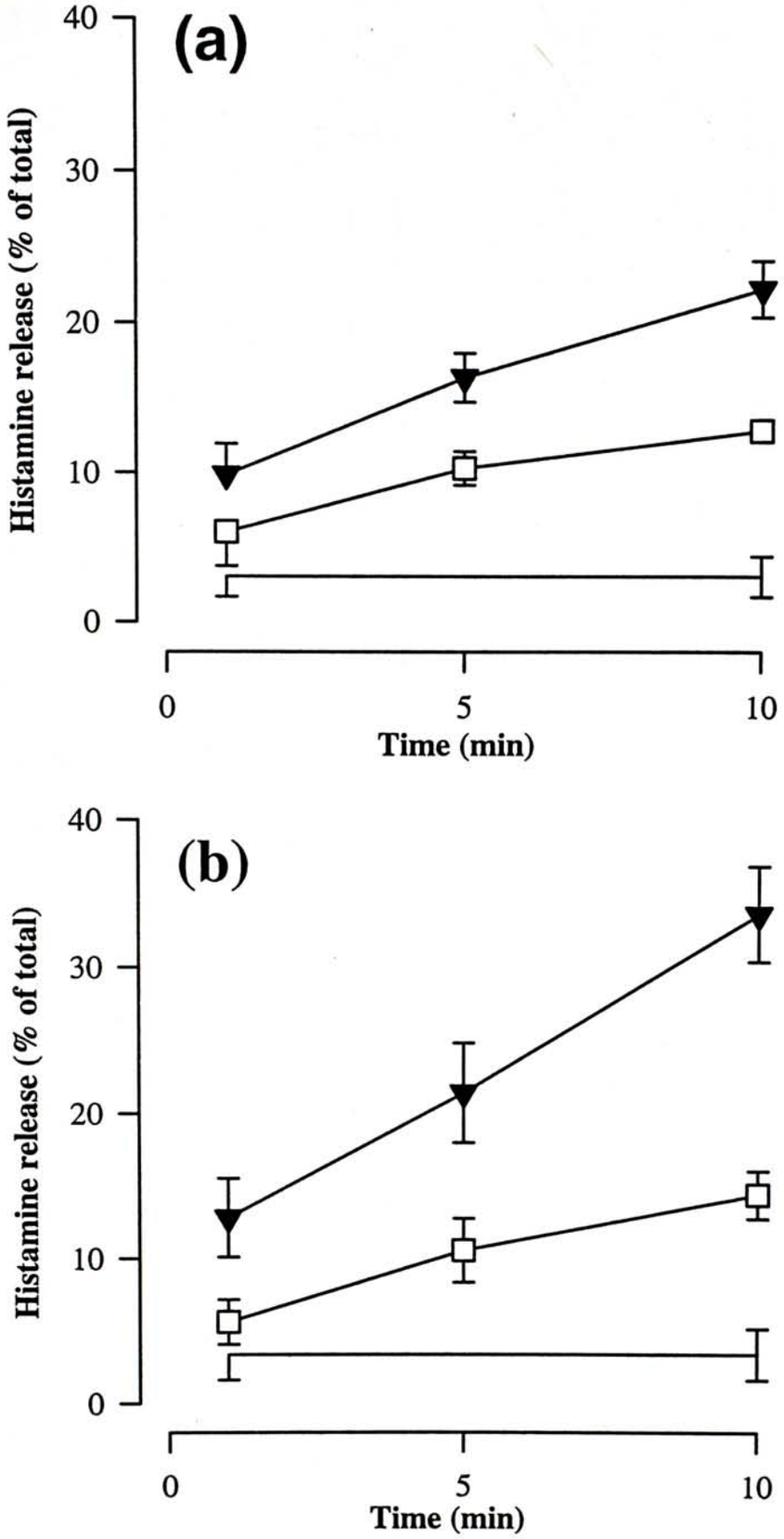

Figure 3.18 Effects of preincubation time with $sPLA_2$-I (0.1 U ml^{-1}) on anti-rat IgE (1/3,000) induced histamine secretion from purified rat peritoneal mast cells. Control cells were incubated with $sPLA_2$-I (□). Anti-rat IgE stimulated cells were pretreated with $sPLA_2$-I for 1, 5 and 10 min and subsequently challenged for 5 min (a) or 10 min (b) (▼). Anti-rat IgE induced histamine release was 3.05 ± 1.36% (a) and 3.38 ± 1.76% (b) (—). Results are given as the means ± SEM for n = 4-6.

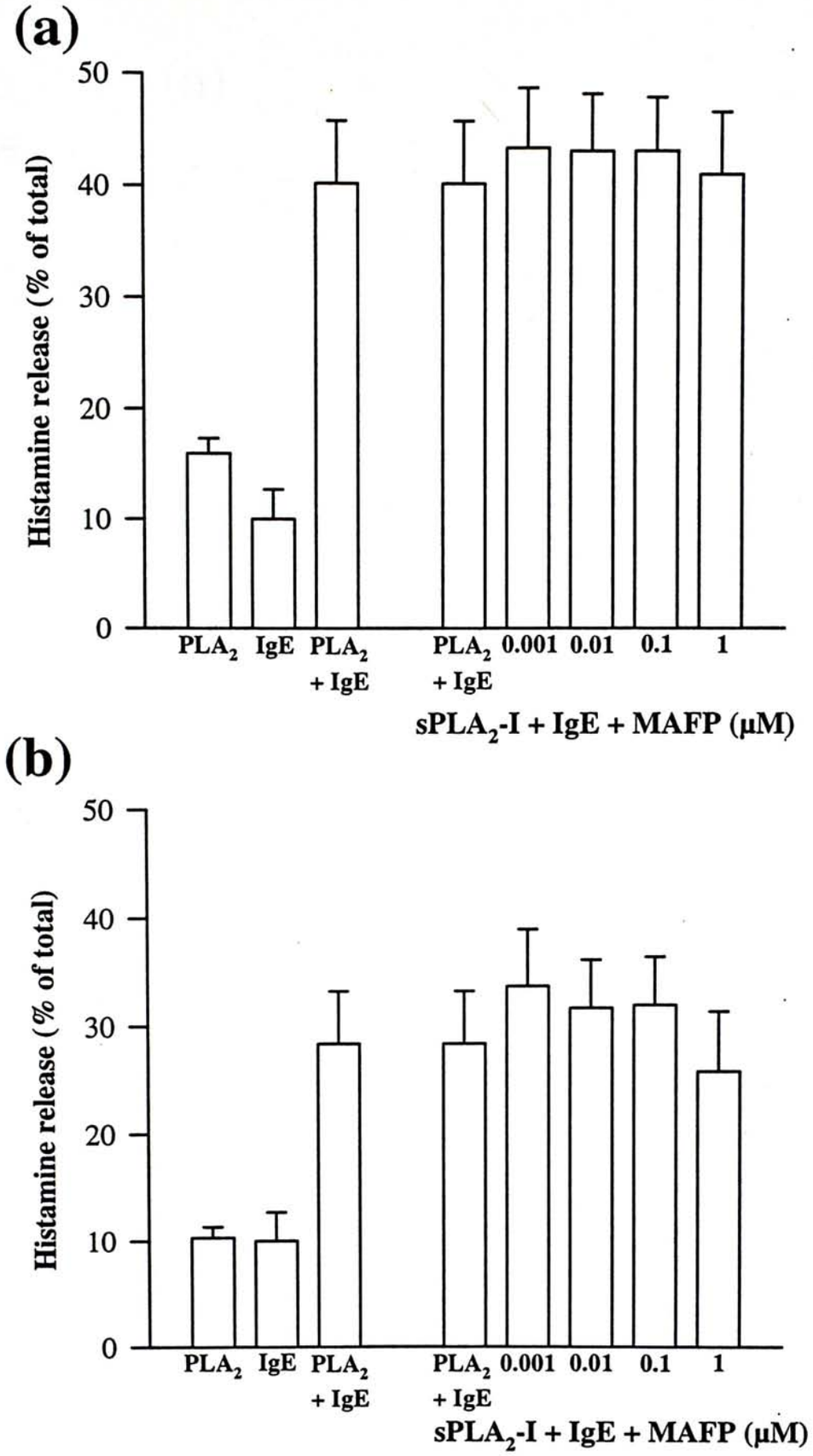

Figure 3.19 (a + b) Effects of MAFP on anti-rat IgE stimulated secretion from $sPLA_2$-I pretreated purified rat peritoneal mast cells. $sPLA_2$-I (0.1 (a) and 0.01 (b) U ml^{-1}) was incubated with MAFP for 30 min at 37°C before the addition of cells. Anti-IgE (1/3,000) induced histamine release was 9.99 ± 2.68%. The spontaneous histamine release was 11.33 ± 0.79%. Results are given as the means ± SEM for n = 5.

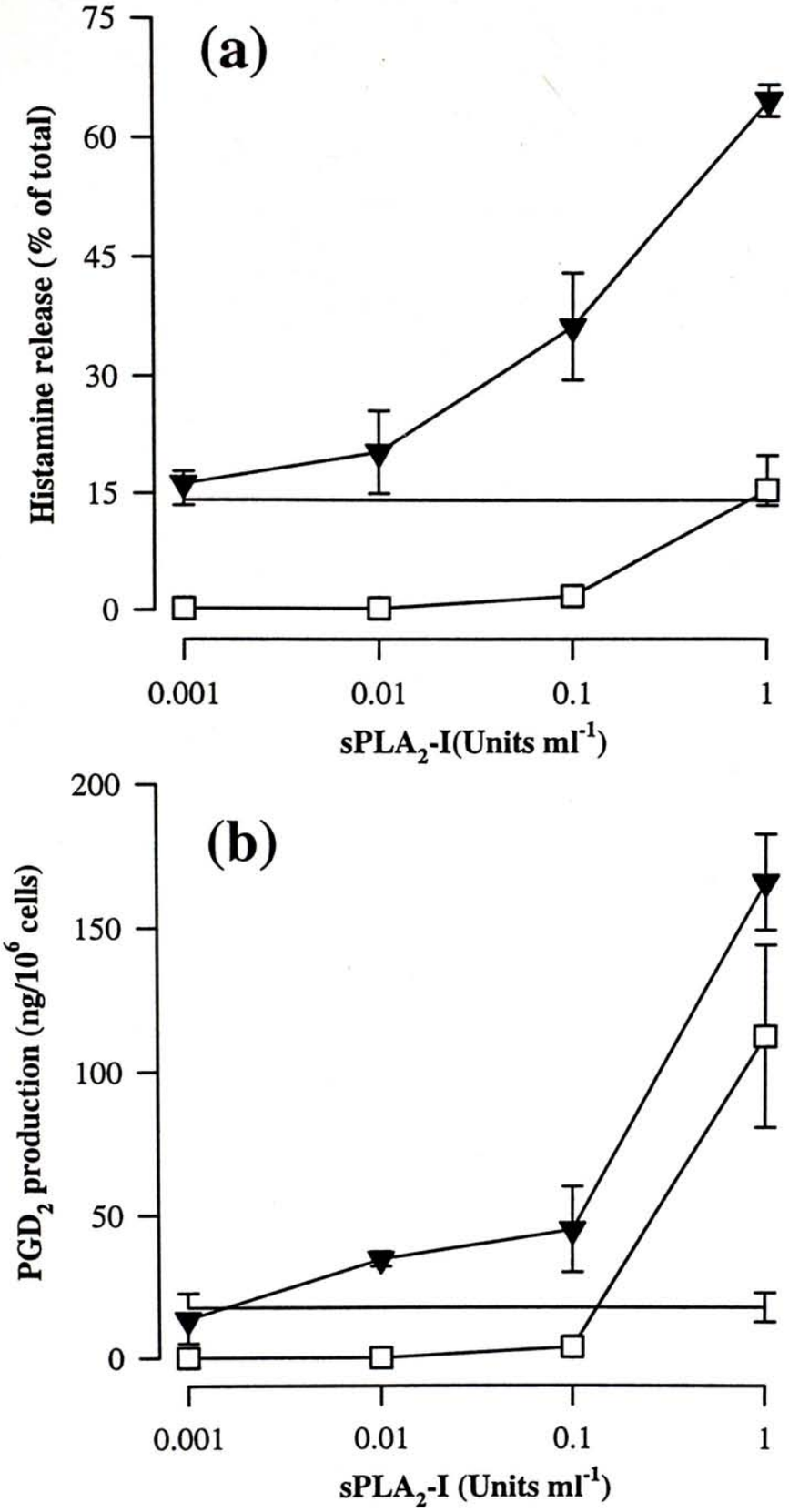

Figure 3.20 (a + b) Effects of $sPLA_2$-I on anti-IgE induced (a) histamine secretion and (b) PGD_2 production from purified rat peritoneal mast cells. Control cells were incubated with $sPLA_2$-I (0.001 to 1 U ml^{-1}) for 20 min (□). Anti-IgE stimulated cells were pretreated with $sPLA_2$-I for 10 min and stimulated for a further 10 min (▼). Anti-rat IgE (1/100) induced histamine release was 14.14 ± 0.67% (—) and PGD_2 production was 17.67 ± 5.12 ng/10^6 cells. The spontaneous histamine release was 11.10 ± 1.72%. Results are given as the means ± SEM for n = 3.

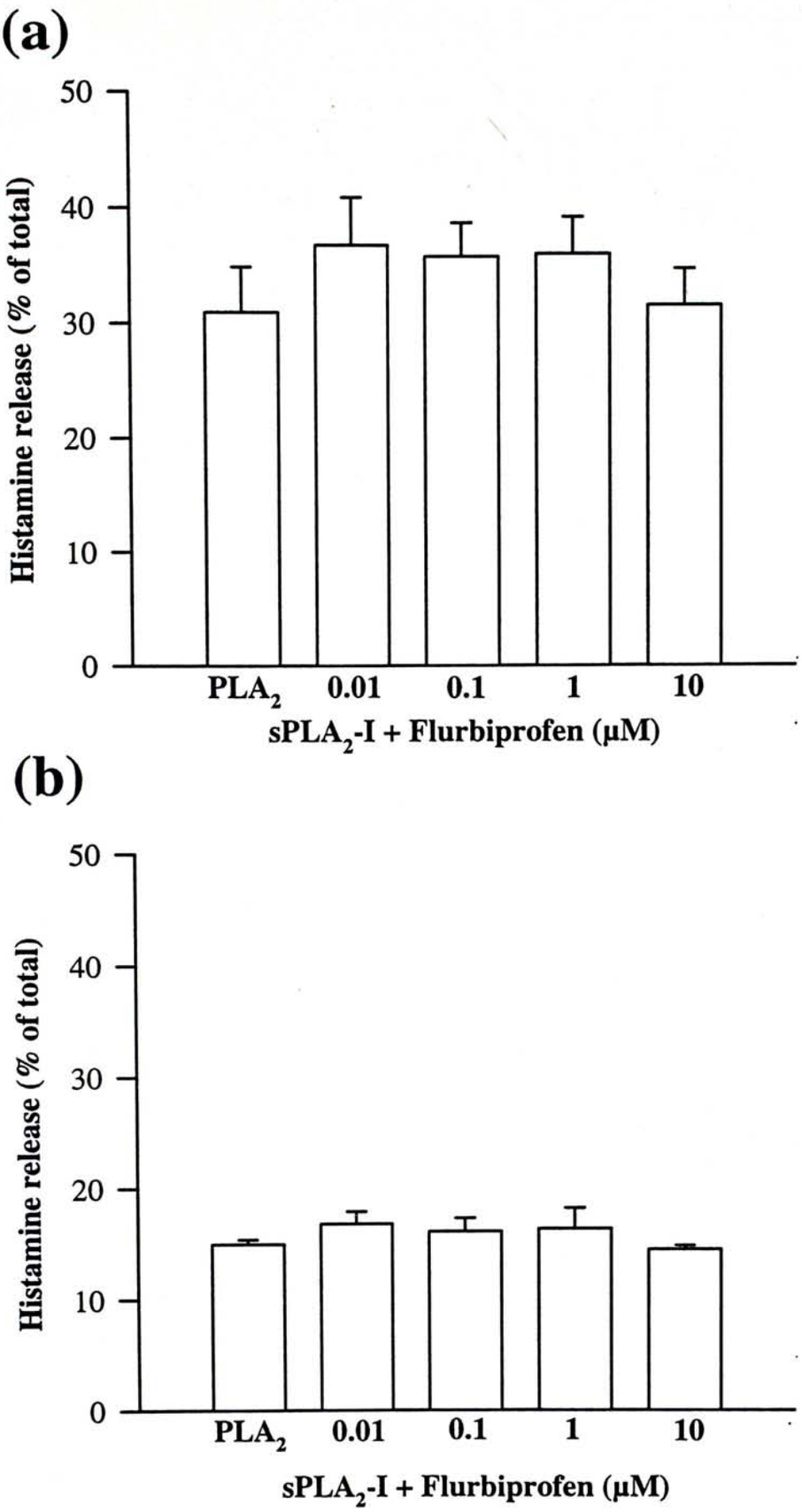

Figure 3.21 (a + b) Effects of flurbiprofen on $sPLA_2$-I induced histamine release from $sPLA_2$-I pretreated purified rat peritoneal mast cells. Cells were preincubated with flurbiprofen for 15 min at 37°C before incubation with $sPLA_2$-I (0.1 U ml^{-1} (a) and 0.01 (b) U ml^{-1}) for 20 min. The spontaneous histamine release was 13.99 ± 1.76%. Results are given as the means ± SEM for n = 4.

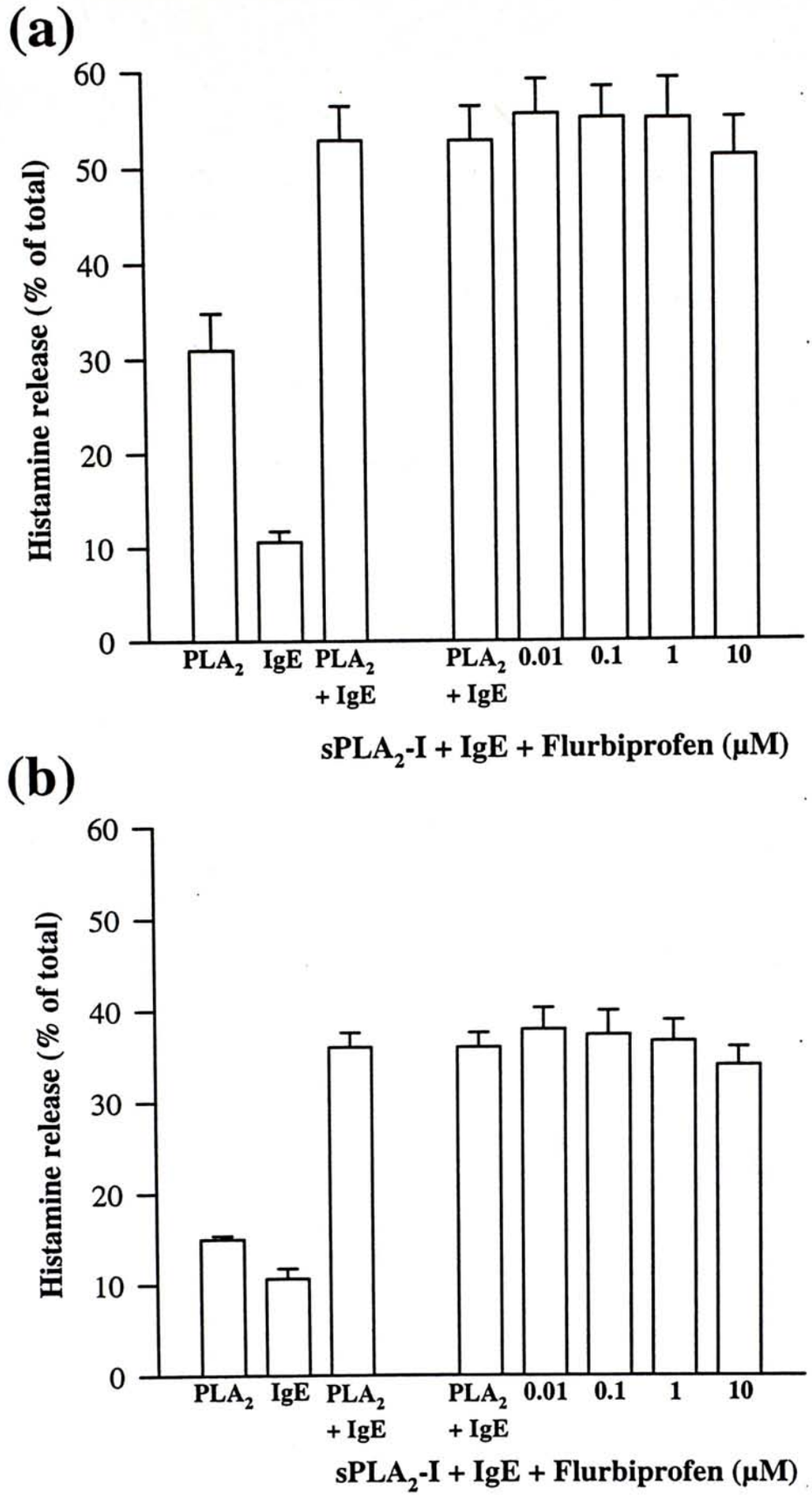

Figure 3.22 (a + b) Effects of flurbiprofen on anti-rat IgE stimulated secretion from $sPLA_2$-I pretreated purified rat peritoneal mast cells. Cells were preincubated with flurbiprofen for 15 min at 37°C before incubation with $sPLA_2$-I (0.1 U ml^{-1} (a) and 0.01 (b) U ml^{-1}) for 10 min. Cells were stimulated with anti-IgE (1/3,000) for a further 10 min. Anti-IgE induced histamine release was 10.61 ± 1.12%. The spontaneous histamine release was 13.99 ± 1.76%. Results are given as the means ± SEM for n =4.

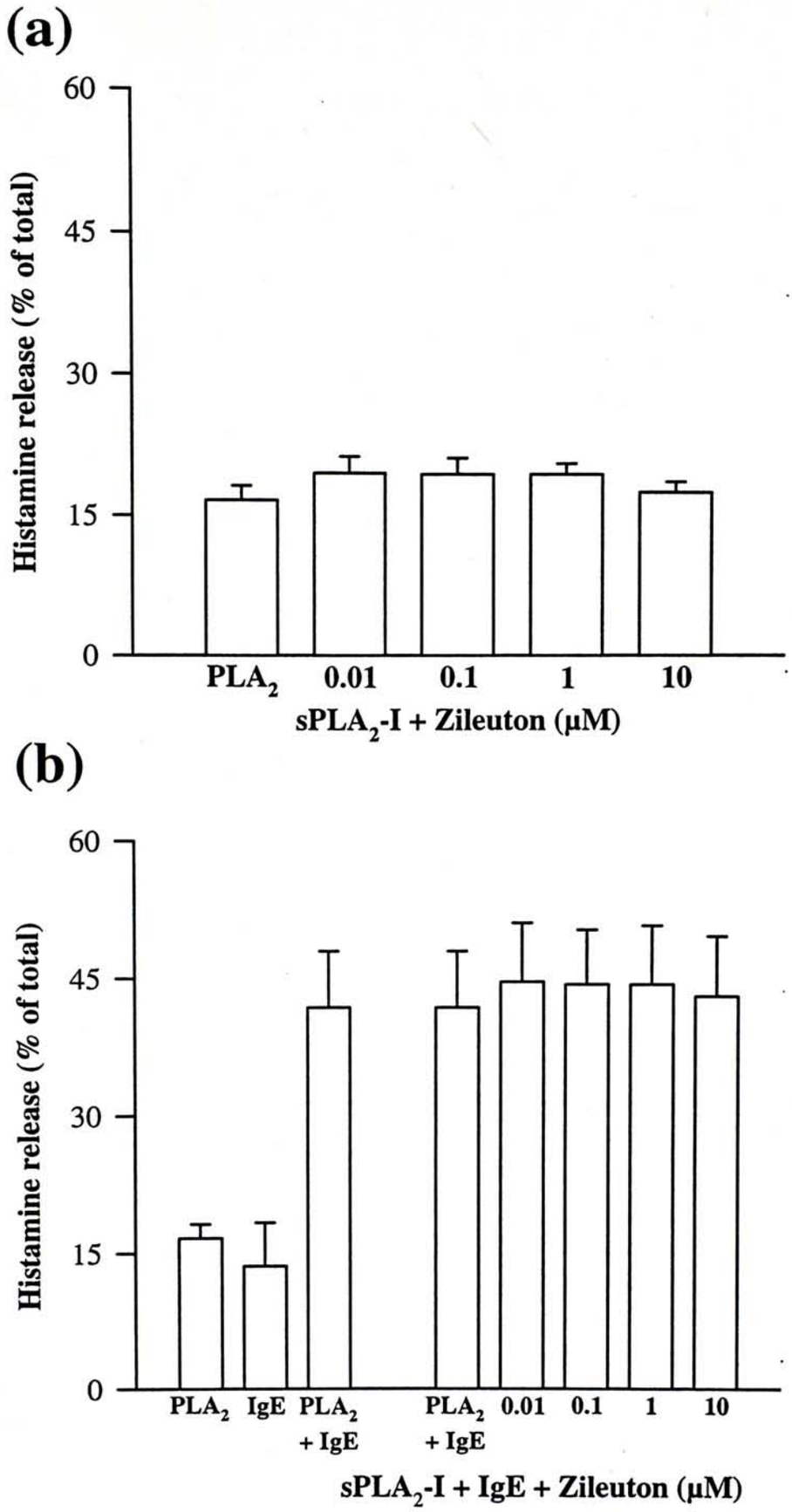

Figure 3.23 (a + b) Effects of zileuton on (a) $sPLA_2$-I induced histamine release and (b) anti-rat IgE stimulated secretion from $sPLA_2$-I pretreated purified rat peritoneal mast cells. Cells were preincubated with zileuton for 15 min at 37°C before incubation with $sPLA_2$-I (0.01 U ml^{-1}). Cells were stimulated with anti-IgE (1/3,000) for a further 10 min. Anti-IgE induced histamine release was 13.58 ± 4.77%. The spontaneous histamine release was 12.00 ± 0.95%. Results are given as the means ± SEM for n =5.

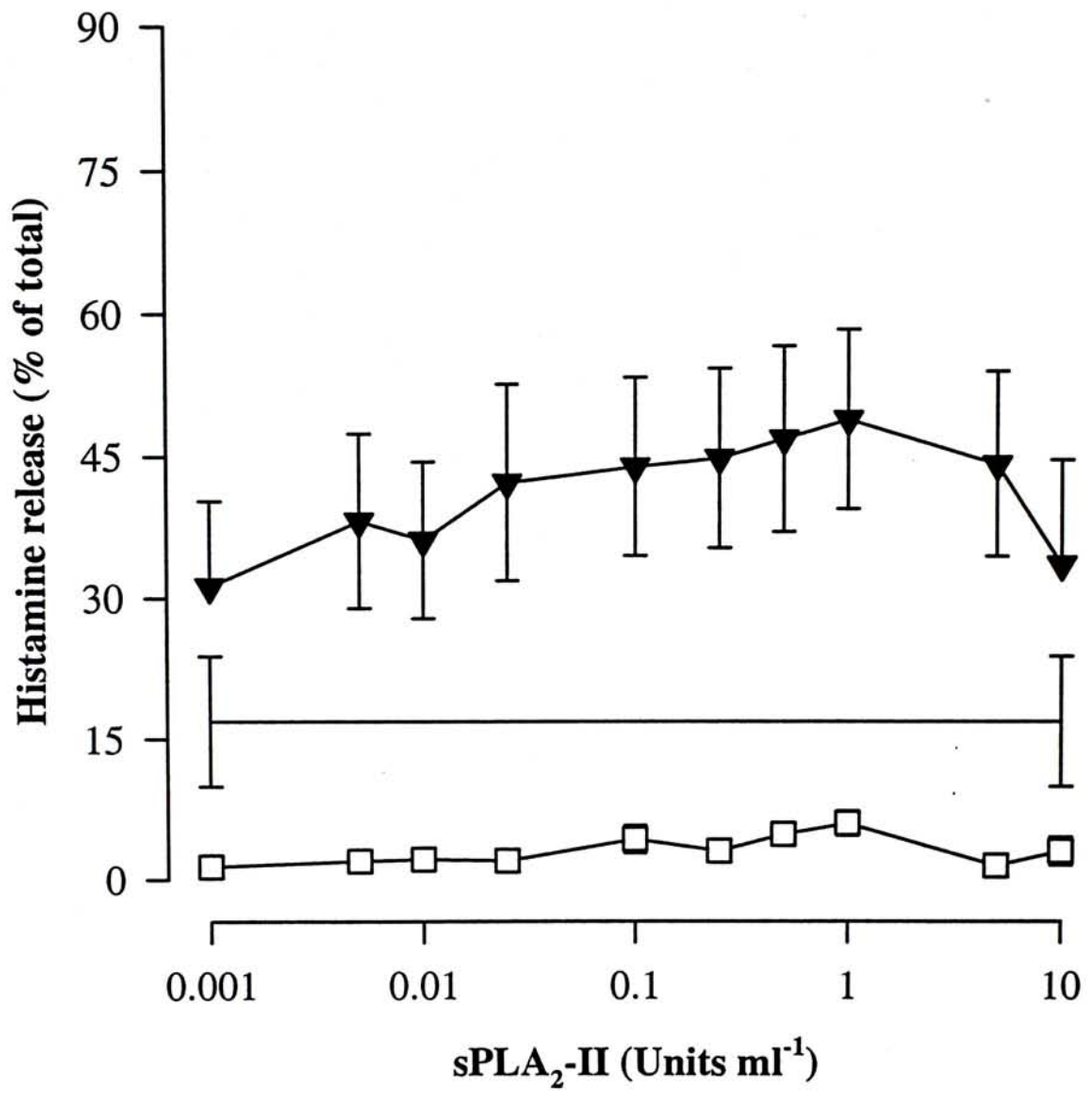

Figure 3.24 Effects of $sPLA_2$-II on anti-rat IgE induced histamine secretion from rat peritoneal mast cells. Control cells were incubated with $sPLA_2$-II (0.001 to 10 Uml^{-1}) for 20 min (□). Anti-rat IgE stimulated cells were pretreated with $sPLA_2$-II for 10 min and stimulated for a further 10 min with anti-rat IgE (▼). Anti-rat IgE (1/1,000) induced histamine release was 16.89 ± 6.97% (—). The spontaneous histamine release was 7.55 ± 0.84%. Results are given as the means ± SEM for n = 5-6.

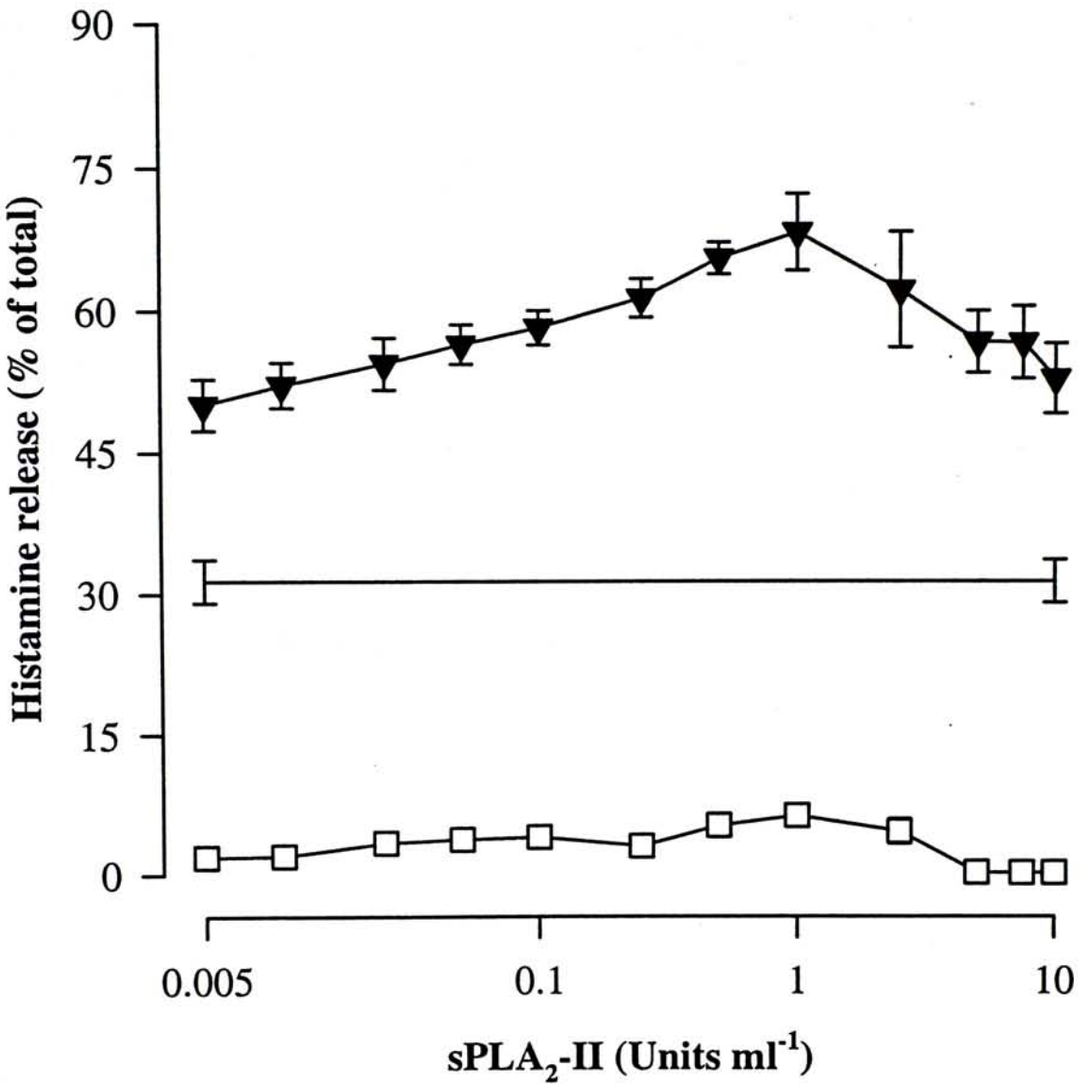

Figure 3.25 Effects of $sPLA_2$-II on anti-rat IgE induced histamine secretion from purified rat peritoneal mast cells. Control cells were incubated with $sPLA_2$-II (0.005 to 10 U ml^{-1}) for 20 min (□). Anti-rat IgE stimulated cells were pretreated with $sPLA_2$-II for 10 min and stimulated for a further 10 min (▼). Anti-rat IgE (1/100) induced histamine release was 31.37 ± 2.30% (—). The spontaneous histamine release was 7.73 ± 0.48%. Results are given as the means ± SEM for n = 5-6.

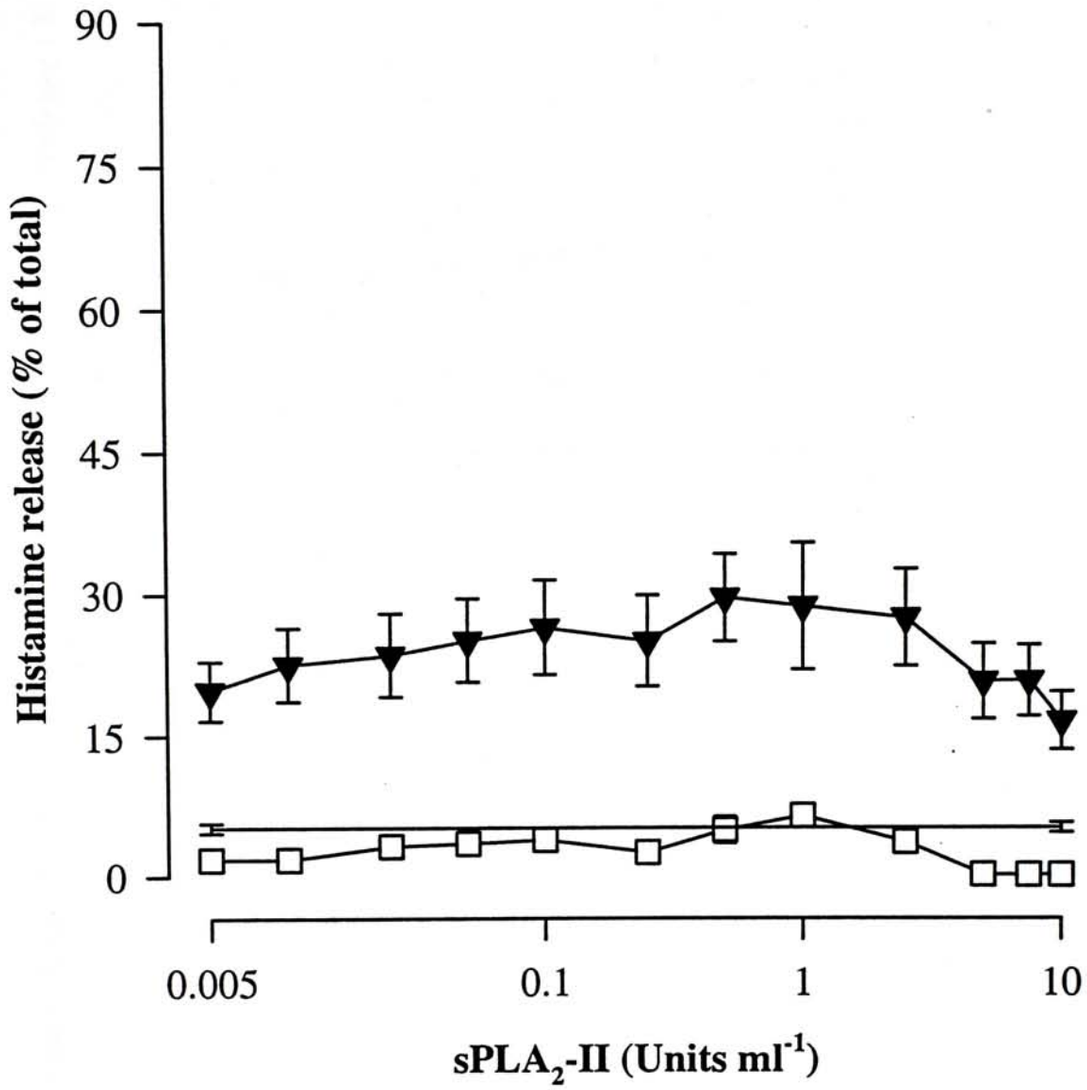

Figure 3.26 Effects of $sPLA_2$-II on anti-rat IgE induced histamine secretion from purified rat peritoneal mast cells. Control cells were incubated with $sPLA_2$-II (0.005 to 10 U ml^{-1}) for 20 min (□). Anti-rat IgE stimulated cells were pretreated with $sPLA_2$-II for 10 min and stimulated for a further 10 min (▼). Anti-rat IgE (1/10,000) induced histamine release was 5.17 ± 0.54% (—). The spontaneous histamine release was 7.73 ± 0.48%. Results are given as the means ± SEM for n = 4-7.

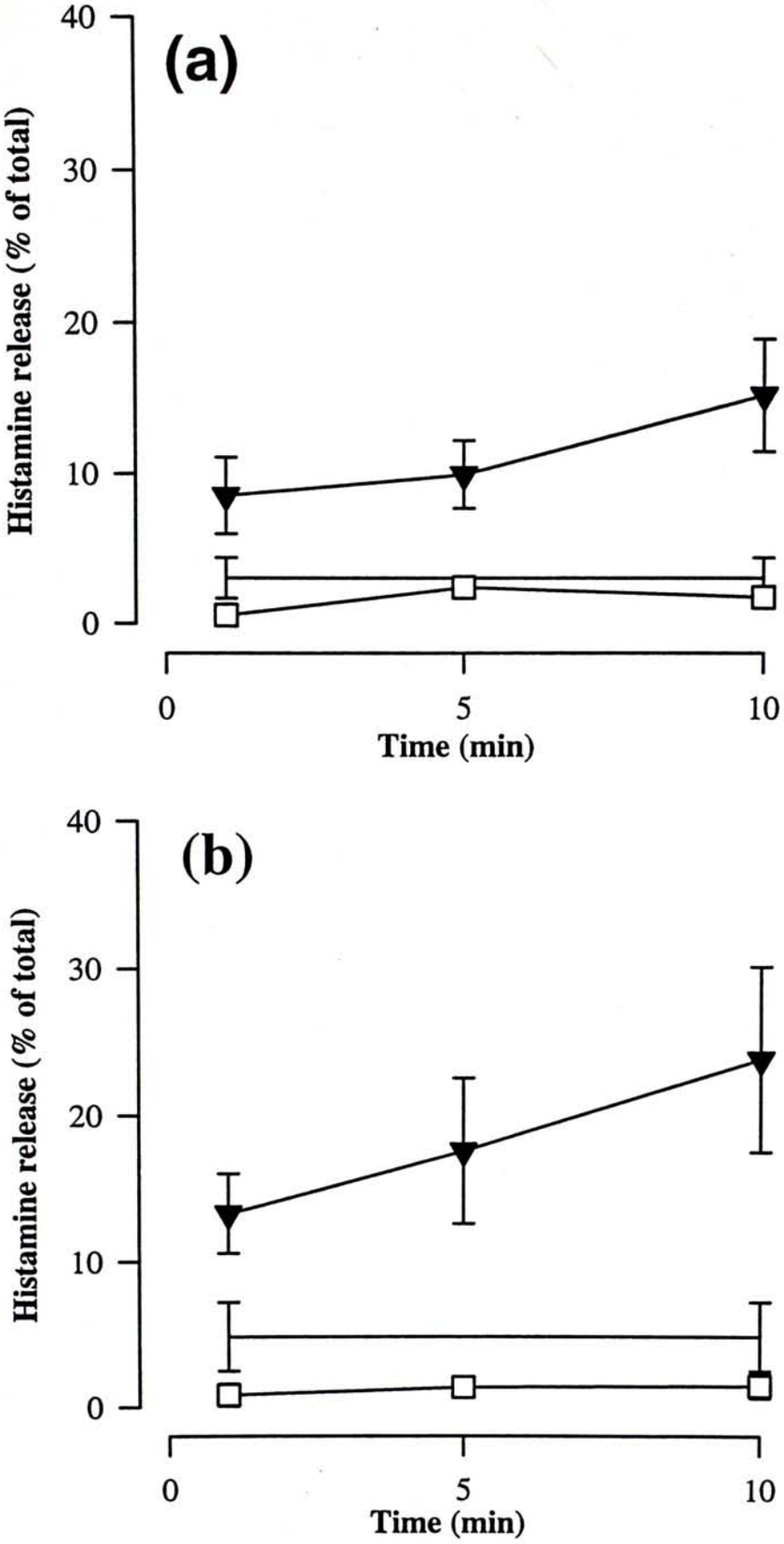

Figure 3.27 Effects of preincubation time with $sPLA_2$-II (0.1 U ml^{-1}) on anti-rat IgE (1/3,000) induced histamine secretion from purified rat peritoneal mast cells. Control cells were incubated with $sPLA_2$-II (□). Anti-rat IgE stimulated cells were pretreated with $sPLA_2$-I for 1, 5 and 10 min and subsequently challenged for 5 min (a) or 10 min (b) (▼). Anti-rat IgE induced histamine release was (a) 3.05 ± 1.36% and (b) 3.38 ± 1.76% (—). Results are given as the means ± SEM for n = 4.

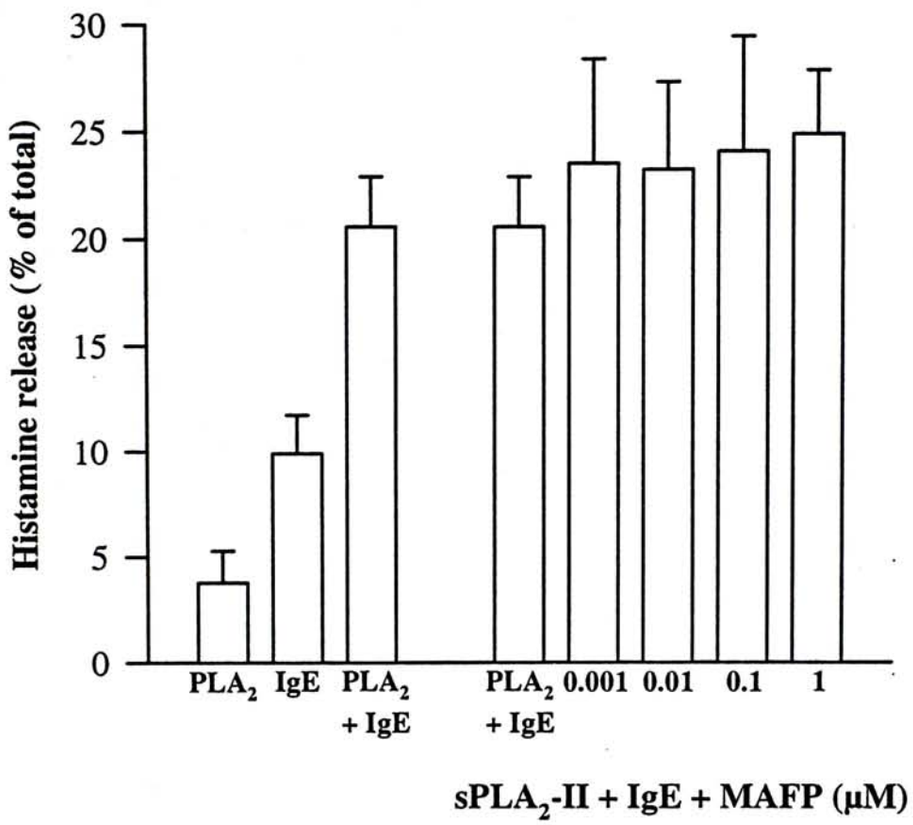

Figure 3.28 Effects of MAFP on anti-rat IgE stimulated secretion from $sPLA_2$-II pretreated purified rat peritoneal mast cells. $sPLA_2$-II (1 U ml^{-1}) was incubated with MAFP for 30 min at 37°C before the addition of cells. Anti-rat IgE (1/3,000) induced histamine release was 9.92 ± 1.82%. The spontaneous histamine release was 10.77 ± 0.66%. Results are given as the means ± SEM for n = 3-4

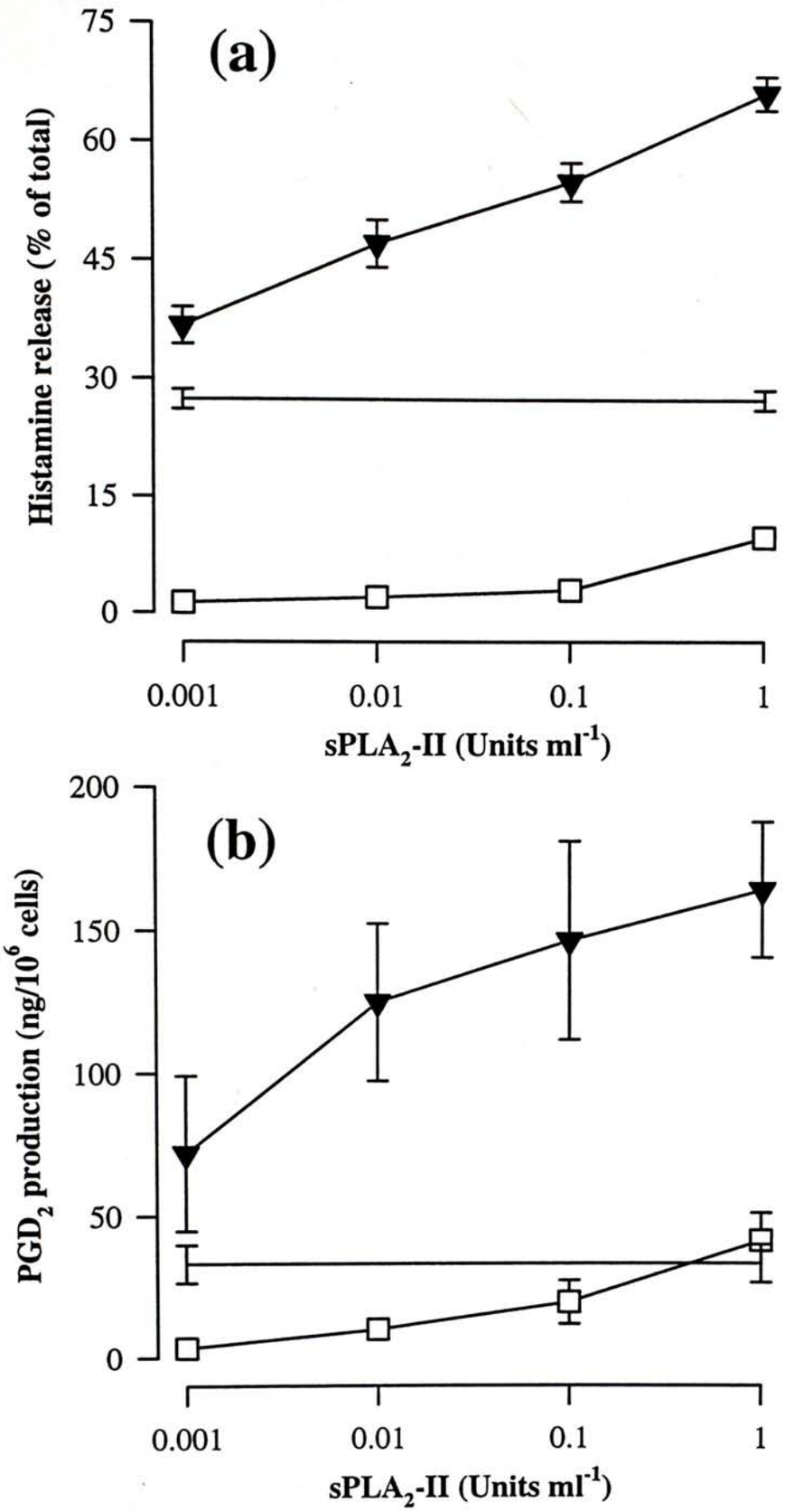

Figure 3.29 (a + b) Effects of $sPLA_2$-II on anti-rat IgE induced (a) histamine secretion and (b) PGD_2 production from purified rat peritoneal mast cells. Control cells were incubated with $sPLA_2$-II (0.001 to 1 U ml^{-1}) for 20 min (□). Anti-rat IgE stimulated cells were pretreated with $sPLA_2$-II for 10 min and stimulated for a further 10 min (▼). Anti-rat IgE (1/100) induced histamine release was 27.33 ± 1.29% (—) and PGD_2 production was 33.19 ± 6.73 ng/10^6 cells. The spontaneous histamine release was 10.70 ± 1.77%. Results are given as the means ± SEM for n = 4-5.

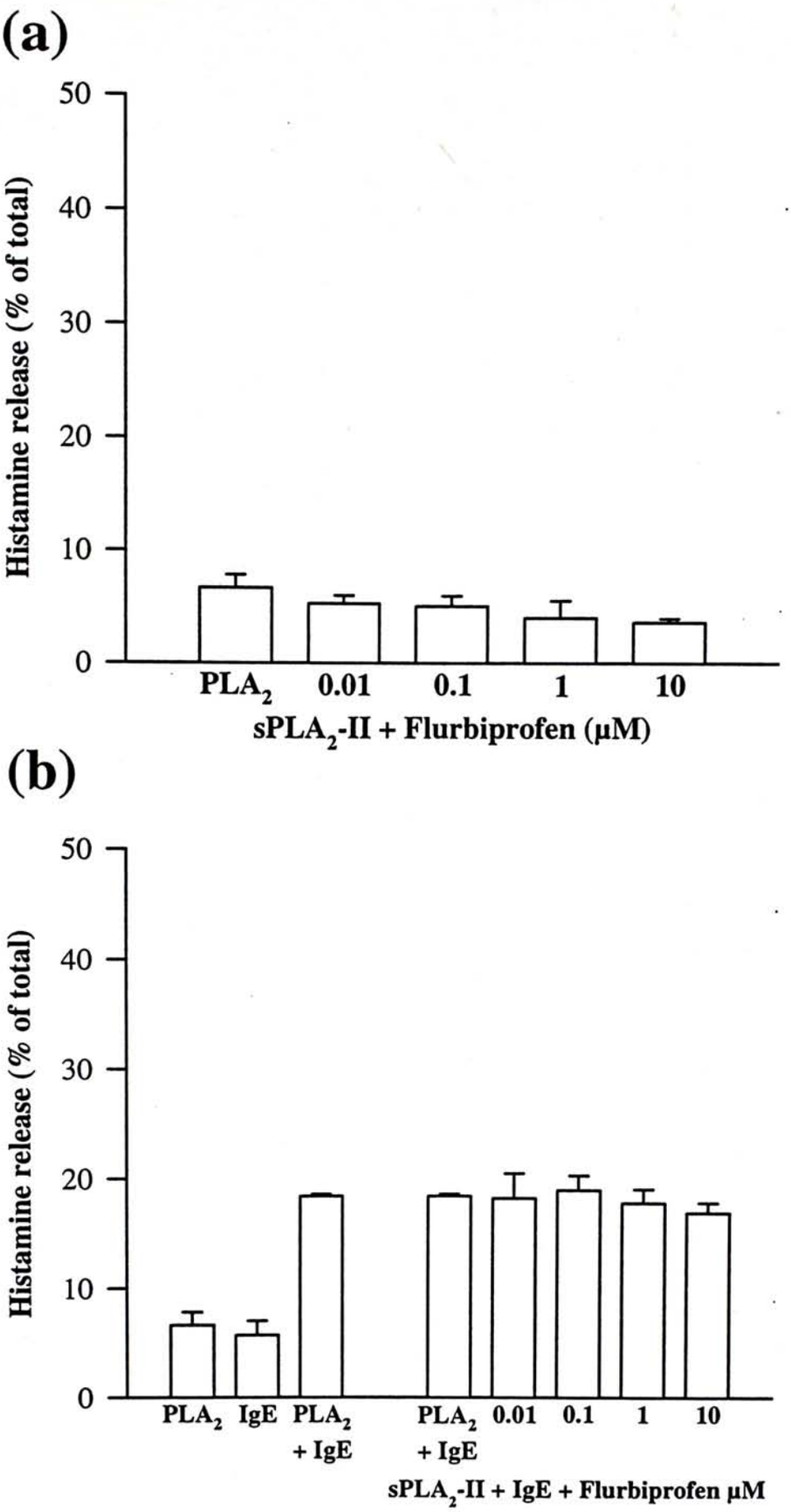

Figure 3.30 (a + b) Effects of flurbiprofen on (a) $sPLA_2$-II induced histamine release and (b) anti-rat IgE stimulated secretion from $sPLA_2$-II pretreated purified rat peritoneal mast cells. Cells were preincubated with flurbiprofen for 15 min at 37°C before incubation with $sPLA_2$-II (0.01 U ml^{-1}). Cells were stimulated with anti-IgE (1/3,000) for a further 10 min. Anti-IgE induced histamine release was 5.72 ± 1.30%. The spontaneous histamine release was 11.88 ± 0.52%. Results are given as the means ± SEM for n = 3.

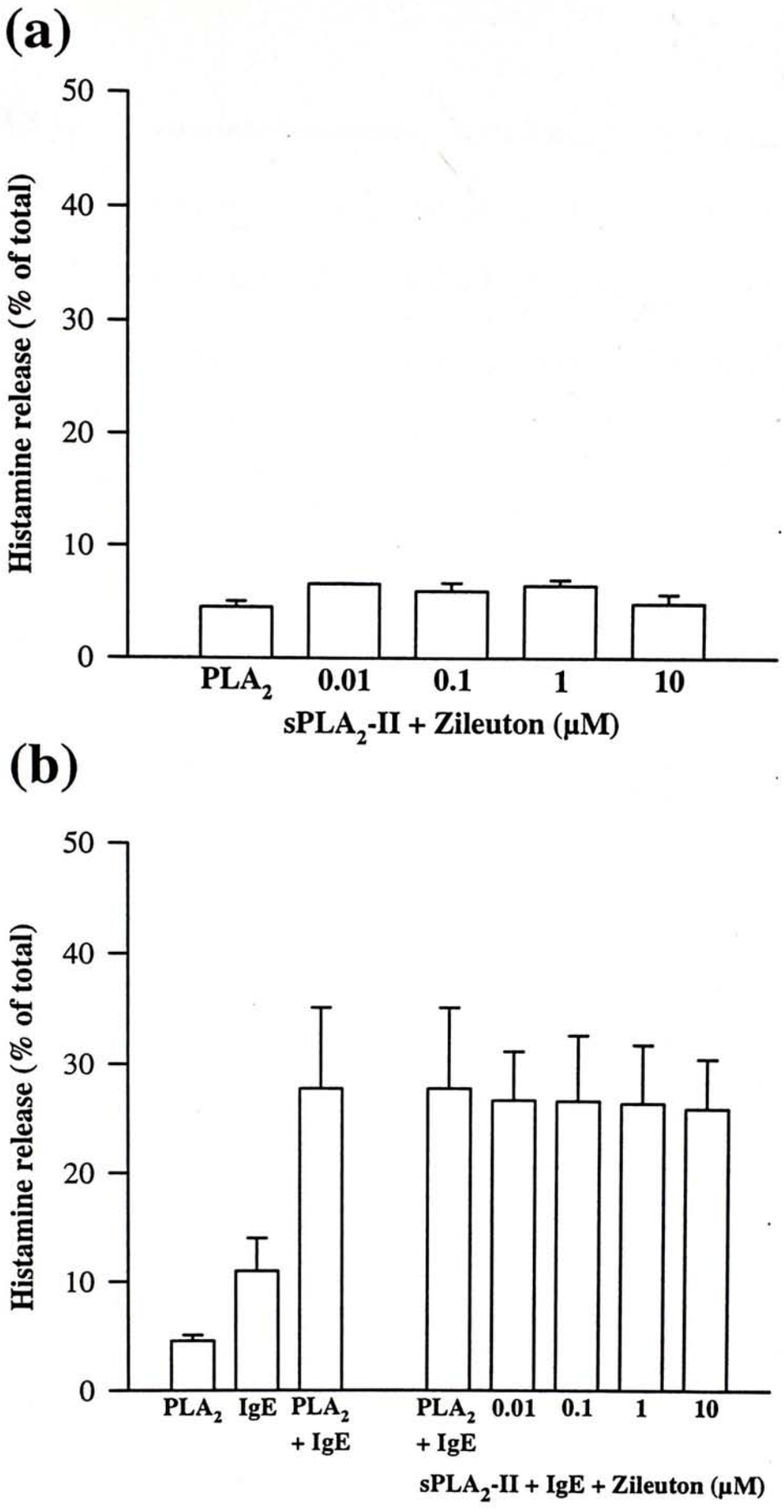

Figure 3.31 (a + b) Effects of zileuton on (a) $sPLA_2$-II induced histamine release and (b) anti-rat IgE stimulated secretion from $sPLA_2$-II pretreated purified rat peritoneal mast cells. Cells were preincubated with zileuton for 15 min at 37°C before incubation with $sPLA_2$-II (0.01 U ml^{-1}). Cells were stimulated with anti-IgE (1/3,000) for a further 10 min. Anti-IgE induced histamine release was 8.05 ± 1.36%. The spontaneous histamine release was 10.95 ± 1.69%. Results are given as the means ± SEM for n = 3.

Table 3.3 Comparison between histamine release induced by anti-rat IgE from $sPLA_2$-I pretreated rat peritoneal mast cells with the calculated combined histamine release induced by $sPLA_2$-I and anti-rat IgE. Anti-rat IgE induced histamine release was 7.48 ± 3.66%. * = $p \leq 0.05$ as compared with the calculated combined value. Values are means ± SEM for n = 3-5.

	Histamine release (% of total)	
$sPLA_2$-I	**[$sPLA_2$-I + IgE]**	**[$sPLA_2$-I] + [IgE]**
10 Units ml^{-1}	80.70 ± 1.46	89.26 ± 5.22
5.0 Units ml^{-1}	78.36 ± 1.72	82.65 ± 4.77
1.0 Unit ml^{-1}	62.95 ± 4.45	46.29 ± 7.40
0.5 Units ml^{-1}	54.18 ± 6.29*	25.21 ± 3.78
0.25 Units ml^{-1}	45.82 ± 6.34*	16.49 ± 2.59
0.1 Units ml^{-1}	37.51 ± 8.62*	15.00 ± 4.24
0.05 Units ml^{-1}	34.00 ± 6.71*	13.02 ± 3.70
0.01 Units ml^{-1}	21.63 ± 8.00	6.35 ± 1.96

[$sPLA_2$-I + IgE] = histamine release induced by anti-rat IgE from $sPLA_2$-I pretreated cells as observed in the experiment.

[$sPLA_2$-I] + [IgE] = combined histamine release, calculated by adding the release induced by $sPLA_2$-I alone to that induced by anti-rat IgE alone for each individual experiment.

Table 3.4 Comparison between histamine release induced by anti-rat IgE from $sPLA_2$-I pretreated purified rat peritoneal mast cells with the calculated combined histamine release induced by $sPLA_2$-I and anti-rat IgE. Anti-rat IgE induced histamine release was 33.57 ± 1.70%. ** = $p \leq 0.01$ and * = $p \leq 0.05$ as compared with the calculated combined value. Values are means ± SEM for n = 5-6.

	Histamine release (% of total)	
$sPLA_2$-I	**[$sPLA_2$-I + IgE]**	**[$sPLA_2$-I] + [IgE]**
10 Units ml^{-1}	81.08 ± 0.66	101.27 ± 2.40
7.5 Units ml^{-1}	81.39 ± 0.93	99.57 ± 3.45
5.0 Units ml^{-1}	81.02 ± 0.68	99.49 ± 3.14
2.5 Units ml^{-1}	80.65 ± 0.86	89.36 ± 2.11
1.0 Unit ml^{-1}	75.88 ± 0.90*	66.41 ± 2.78
0.5 Units ml^{-1}	73.09 ± 1.63**	52.83 ± 2.64
0.25 Units ml^{-1}	69.72 ± 3.16**	48.80 ± 1.98
0.1 Units ml^{-1}	66.76 ± 2.76**	50.00 ± 1.22
0.05 Units ml^{-1}	65.82 ± 2.54**	45.50 ± 1.22
0.025 Units ml^{-1}	61.17 ± 3.64**	43.14 ± 2.02
0.01 Units ml^{-1}	56.58 ± 2.73**	41.74 ± 2.51
0.005 Units ml^{-1}	53.94 ± 2.72**	40.96 ± 2.91

[$sPLA_2$-I + IgE] = histamine release induced by anti-rat IgE from $sPLA_2$-I pretreated cells as observed in the experiment.

[$sPLA_2$-I] + [IgE] = combined histamine release, calculated by adding the release induced by $sPLA_2$-I alone to that induced by anti-rat IgE alone for each individual experiment.

Table 3.5 Comparison between histamine release induced by anti-rat IgE from $sPLA_2$-I pretreated purified rat peritoneal mast cells with the calculated combined histamine release induced by $sPLA_2$-I and anti-rat IgE. Anti-rat IgE induced histamine release was 5.85 ± 0.76%. ** = $p \leq 0.01$ and * = $p \leq 0.05$ as compared with the calculated combined value. Values are means ± SEM for n = 4-7.

Histamine release (% of total)

$sPLA_2$-I	[$sPLA_2$-I + IgE]	[$sPLA_2$-I] + [IgE]
10 Units ml^{-1}	76.47 ± 1.09	72.05 ± 2.48
7.5 Units ml^{-1}	77.96 ± 1.09	71.61 ± 2.28
5.0 Units ml^{-1}	77.62 ± 1.05	70.84 ± 2.54
2.5 Units ml^{-1}	67.70 ± 4.64	60.69 ± 2.76
1.0 Unit ml^{-1}	52.76 ± 6.04*	37.68 ± 3.76
0.5 Units ml^{-1}	45.32 ± 4.96**	24.87 ± 0.86
0.25 Units ml^{-1}	38.91 ± 0.25*	21.71 ± 0.80
0.1 Units ml^{-1}	40.69 ± 5.56*	18.83 ± 0.47
0.05 Units ml^{-1}	36.45 ± 6.43*	14.96 ± 1.07
0.025 Units ml^{-1}	33.04 ± 5.18*	14.98 ± 1.24
0.01 Units ml^{-1}	27.26 ± 4.56*	12.49 ± 0.67
0.005 Units ml^{-1}	27.54 ± 4.87**	11.89 ± 0.87

[$sPLA_2$-I + IgE] = histamine release induced by anti-rat IgE from $sPLA_2$-I pretreated cells as observed in the experiment.

[$sPLA_2$-I] + [IgE] = combined histamine release, calculated by adding the release induced by $sPLA_2$-I alone to that induced by anti-rat IgE alone for each individual experiment.

Table 3.6 Effects of 12-epi-scalaradial, *p*-BPB and MAFP on histamine release induced by anti-rat IgE from purified rat peritoneal mast cells. Cells were preincubated with 12-epi-scalaradial, *p*-BPB and MAFP for 10 min before activation with anti-rat IgE. ** = $p \leq 0.01$ as compared with the appropriate control. Values are means ± SEM.

Histamine release (% of total)

12-epi-scalaradial μM (n = 13)					
Control	**0.01 μM**	**0.1 μM**	**0.3 μM**	**0.6 μM**	**1 μM**
9.47 ± 1.32	6.78 ± 2.48	5.88±1.46**	2.34±0.85**	0.37±0.21**	0.1±0.07**
***p*-bromophenacyl bromide μM (n = 9)**					
Control	**0.01 μM**	**0.1 μM**	**1 μM**	**10 μM**	
8.09 ± 1.03	8.70 ± 1.07	6.43±1.24**	2.22±0.68**	0.38±0.15**	
Methyl arachidonyl fluorophosphonate μM (n = 9)					
Control	**0.001 μM**	**0.01 μM**	**0.1 μM**	**1 μM**	
9.96 ± 1.60	9.94 ± 2.06	8.93 ± 1.77	9.64 ± 1.96	8.08 ± 1.93	

Table 3.7 (a + b) Comparison between histamine release induced by anti-rat IgE from $sPLA_2$-I pretreated purified rat peritoneal mast cells with the calculated combined histamine release induced by $sPLA_2$-I and anti-IgE in the presence of *p*-BPB. $sPLA_2$-I concentrations tested were (a) 0.1 U ml^{-1} and (b) 0.01 U ml^{-1}. ** = $p \leq 0.01$ as compared with the calculated combined value. Values are means ± SEM for n = 3-4.

Table 3.7 a **Histamine release (% of total)**

***p*-BPB**	**[$sPLA_2$-I + IgE]**	**[$sPLA_2$-I] + [IgE]**
0.01 μM	52.06 ± 2.14**	28.20 ± 3.01
0.1 μM	42.53 ± 3.09**	22.24 ± 2.92
1.0 μM	17.35 ± 2.33	12.47 ± 2.87
10 μM	5.21 ± 2.13	3.48 ± 1.90

Table 3.7 b **Histamine release (% of total)**

***p*-BPB**	**[$sPLA_2$-I + IgE]**	**[$sPLA_2$-I] + [IgE]**
0.01 μM	29.47 ± 2.87**	17.73 ± 2.11
0.1 μM	26.29 ± 3.68**	14.53 ± 2.53
1.0 μM	10.91 ± 1.93	7.70 ± 2.46
10 μM	3.33 ± 1.32	1.89 ± 0.87

[$sPLA_2$-I + IgE] = histamine release induced by anti-rat IgE from $sPLA_2$-I pretreated cells in the presence of *p*-BPB as observed in the experiment.

[$sPLA_2$-I] + [IgE] = combined histamine release, calculated by adding the release induced by $sPLA_2$-I alone to that induced by anti-rat IgE alone in the presence of *p*-BPB for each individual experiment.

Table 3.8 (a + b) Comparison between histamine release induced by anti-rat IgE from $sPLA_2$-I pretreated purified rat peritoneal mast cells with the calculated combined histamine release induced by $sPLA_2$-I and anti-rat IgE in the presence of 12-epi-scalaradial. $sPLA_2$-I concentrations tested were (a) 0.1 U ml^{-1} and (b) 0.01 U ml^{-1}. * = $p \leq 0.05$ as compared with the calculated combined value. Values are means ± SEM for n = 3-7.

Table 3.8 a **Histamine release (% of total)**

12-epi-scalaradial	[$sPLA_2$-I + IgE]	[$sPLA_2$-I] + [IgE]
0.1 μM	35.13 ± 3.70*	18.71 ± 1.53
0.3 μM	21.41 ± 3.38*	10.62 ± 2.70
0.6 μM	5.21 ± 1.60	1.73 ± 0.87
1.0 μM	4.10 ± 1.09	0.92 ± 0.63

Table 3.8 b **Histamine release (% of total)**

12-epi-scalaradial	[$sPLA_2$-I + IgE]	[$sPLA_2$-I] + [IgE]
0.1 μM	21.87 ± 2.45*	12.47 ± 1.20
0.3 μM	11.79 ± 2.00*	4.90 ± 2.13
0.6 μM	1.88 ± 0.84	0.55 ± 0.42
1.0 μM	1.36 ± 0.51	0.13 ± 0.13

[$sPLA_2$-I + IgE] = histamine release induced by anti-rat IgE from $sPLA_2$-I pretreated cells in the presence of 12-epi-scalaradial as observed in the experiment.
[$sPLA_2$-I] + [IgE] = combined histamine release, calculated by adding the release induced by $sPLA_2$-I alone to that induced by anti-rat IgE alone in the presence of 12-epi-scalaradial for each individual experiment.

Table 3.9 (a + b) Comparison between (a) histamine release and (b) PGD_2 production, induced by anti-rat IgE from $sPLA_2$-I pretreated purified rat peritoneal mast cells with the calculated combined values from $sPLA_2$-I and anti-rat IgE. Anti-rat IgE induced histamine release was 14.14 ± 0.67% and PGD_2 production was 17.67 ± 5.12 ng/10^6 cells. ** = $p \leq 0.01$ and * = $p \leq 0.05$ as compared with the calculated combined value. Values are means ± SEM for n = 3.

Table 3.9 a **Histamine release (% of total)**

$sPLA_2$-I	[$sPLA_2$-I + IgE]	[$sPLA_2$-I] + [IgE]
1 Unit ml^{-1}	64.99 ± 2.00**	29.55 ± 5.10
0.1 Units ml^{-1}	36.39 ± 6.79*	15.94 ± 1.88
0.01 Units ml^{-1}	20.25 ± 5.30	14.37 ± 0.48
0.001 Units ml^{-1}	16.20 ± 1.59	14.38 ± 0.50

Table 3.9 b **PGD_2 production (ng/10^6 cells)**

$sPLA_2$-I	[$sPLA_2$-I + IgE]	[$sPLA_2$-I] + [IgE]
1 Unit ml^{-1}	166.00 ± 16.69	129.91 ± 27.20
0.1 Units ml^{-1}	45.00 ± 14.98	21.41 ± 8.57
0.01 Units ml^{-1}	34.75 ± 2.61*	17.67 ± 5.12
0.001 Units ml^{-1}	13.50 ± 8.43	17.67 ± 5.12

[$sPLA_2$-I + IgE] = histamine release and PGD_2 production induced by anti-rat IgE from $sPLA_2$-I pretreated cells as observed in the experiment.

[$sPLA_2$-I] + [IgE] = combined histamine release and PGD_2 production, calculated by adding the release/production induced by $sPLA_2$-I alone to that induced by anti-rat IgE alone for each individual experiment.

Table 3.10 Comparison between histamine release induced by anti-rat IgE from $sPLA_2$-II pretreated rat peritoneal mast cells with the combined histamine release induced by $sPLA_2$-II and anti-rat IgE. Anti-rat IgE induced histamine release was 16.89 ± 6.97%. ** = $p \leq 0.01$ and * = $p \leq 0.05$ as compared with the calculated combined value. Values are means ± SEM for n = 5-6.

Histamine release (% of total)

$sPLA_2$-II	**[$sPLA_2$-II + IgE]**	**[$sPLA_2$-II] + [IgE]**
10 Units ml^{-1}	33.56 ± 11.18	21.23 ± 8.59
5.0 Units ml^{-1}	44.26 ± 9.81**	18.29 ± 7.12
1.0 Unit ml^{-1}	49.01 ± 9.49**	22.92 ± 6.40
0.5 Units ml^{-1}	46.94 ± 9.82**	21.77 ± 6.23
0.25 Units ml^{-1}	44.89 ± 9.50**	19.93 ± 6.41
0.1 Units ml^{-1}	44.00 ± 9.45**	21.21 ± 7.49
0.05 Units ml^{-1}	42.32 ± 10.43**	15.53 ± 7.22
0.025 Units ml^{-1}	36.20 ± 8.32*	13.88 ± 6.03
0.01 Units ml^{-1}	38.22 ± 9.26**	18.56 ± 6.97
0.005 Units ml^{-1}	31.23 ± 9.09**	19.58 ± 8.89
0.001 Units ml^{-1}	33.20 ± 9.85	18.50 ± 8.46

[$sPLA_2$-II + IgE] = histamine release induced by anti-rat IgE from $sPLA_2$-II pretreated cells as observed in the experiment.

[$sPLA_2$-II] + [IgE] = combined histamine release, calculated by adding the release induced by $sPLA_2$-II alone to that induced by anti-rat IgE alone for each individual experiment.

Table 3.11 Comparison between histamine release induced by anti-rat IgE from $sPLA_2$-II pretreated purified rat peritoneal mast cells with the calculated combined histamine release induced by $sPLA_2$-II and anti-rat IgE. Anti-rat IgE induced histamine release was 31.37 ± 2.30%. ** = $p \leq 0.01$ as compared with the calculated combined value. Values are means ± SEM for n = 5-6.

	Histamine release (% of total)	
$sPLA_2$-II	**[$sPLA_2$-II + IgE]**	**[$sPLA_2$-II] + [IgE]**
10 Units ml^{-1}	52.96 ± 3.68**	30.51 ± 2.24
7.5 Units ml^{-1}	56.76 ± 3.80**	33.18 ± 1.92
5.0 Units ml^{-1}	56.82 ± 3.26**	32.17 ± 2.23
2.5 Units ml^{-1}	62.32 ± 6.09**	34.76 ± 3.20
1.0 Unit ml^{-1}	68.38 ± 4.03**	37.64 ± 3.13
0.5 Units ml^{-1}	65.66 ± 1.67**	35.80 ± 2.59
0.25 Units ml^{-1}	61.47 ± 2.04**	34.94 ± 2.78
0.1 Units ml^{-1}	58.30 ± 1.80**	34.58 ± 2.70
0.05 Units ml^{-1}	56.53 ± 2.08**	34.31 ± 2.64
0.025 Units ml^{-1}	54.48 ± 2.72**	34.23 ± 2.56
0.01 Units ml^{-1}	52.18 ± 2.38**	32.50 ± 2.30
0.005 Units ml^{-1}	50.09 ± 2.73**	32.38 ± 2.60

[$sPLA_2$-II + IgE] = histamine release induced by anti-rat IgE from $sPLA_2$-II pretreated cells as observed in the experiment.

[$sPLA_2$-II] + [IgE] = combined histamine release, calculated by adding the release induced by $sPLA_2$-II alone to that induced by anti-rat IgE alone for each individual experiment.

Table 3.12 Comparison between histamine release induced by anti-rat IgE from $sPLA_2$-II pretreated purified rat peritoneal mast cells with the calculated combined histamine release induced by $sPLA_2$-II and anti-rat IgE. Anti-rat IgE induced histamine release was 5.17 ± 0.54%. ** = $p \leq 0.01$ and * = $p \leq 0.05$ as compared with the calculated combined value. Values are means ± SEM for n = 4-7.

Histamine release (% of total)

$sPLA_2$-II	**[$sPLA_2$-II + IgE]**	**[$sPLA_2$-II] + [IgE]**
10 Units ml^{-1}	16.54 ± 3.06**	5.30 ± 0.52
7.5 Units ml^{-1}	20.85 ± 3.80**	5.45 ± 0.64
5.0 Units ml^{-1}	20.77 ± 4.02**	5.49 ± 0.68
2.5 Units ml^{-1}	27.55 ± 5.14**	9.03 ± 1.47
1.0 Unit ml^{-1}	28.78 ± 6.73*	13.02 ± 1.97
0.5 Units ml^{-1}	29.70 ± 4.62*	10.12 ± 2.00
0.25 Units ml^{-1}	25.11 ± 4.84**	7.74 ± 0.90
0.1 Units ml^{-1}	25.56 ± 5.04**	9.06 ± 0.84
0.05 Units ml^{-1}	25.20 ± 4.45**	8.69 ± 0.76
0.025 Units ml^{-1}	23.61 ± 4.42**	8.38 ± 1.01
0.01 Units ml^{-1}	22.55 ± 3.91**	7.00 ± 0.82
0.005 Units ml^{-1}	19.79 ± 3.16**	7.00 ± 0.68

[$sPLA_2$-II + IgE] = histamine release induced by anti-rat IgE from $sPLA_2$-II pretreated cells as observed in the experiment.

[$sPLA_2$-II] + [IgE] = combined histamine release, calculated by adding the release induced by $sPLA_2$-II alone to that induced by anti-rat IgE alone for each individual experiment.

Table 3.13 Comparison between histamine release induced by anti-rat IgE from $sPLA_2$-II pretreated purified rat peritoneal mast cells with the calculated combined histamine release induced by $sPLA_2$-II and anti-rat IgE in the presence of *p*-BPB. $sPLA_2$-II concentration tested was 1 U ml^{-1}. * = $p \leq 0.05$ as compared with the calculated combined value. Values are means ± SEM for n = 5.

	Histamine release (% of total)	
***p*-BPB**	**[$sPLA_2$-II + IgE]**	**[$sPLA_2$-II] + [IgE]**
0.01 μM	32.46 ± 1.89*	19.82 ± 1.98
0.1 μM	23.50 ± 3.31*	16.97 ± 1.96
1.0 μM	9.23 ± 1.27	6.59 ± 1.35
10 μM	1.86 ± 0.67	0.52 ± 0.32

[$sPLA_2$-II + IgE] = histamine release induced by anti-rat IgE from $sPLA_2$-II pretreated cells in the presence of *p*-BPB as observed in the experiment.
[$sPLA_2$-II] + [IgE] = combined histamine release, calculated by adding the release induced in the presence of *p*-BPB by $sPLA_2$-II alone to that induced anti-rat IgE alone for each individual experiment.

Table 3.14 Comparison between histamine release induced by anti-rat IgE from $sPLA_2$-II pretreated purified rat peritoneal mast cells with the calculated combined histamine release induced by $sPLA_2$-II and anti-rat IgE in the presence of 12-epi-scalaradial. $sPLA_2$-II concentration tested was 1 U ml^{-1}. ** = $p \leq 0.01$ as compared with the calculated combined value. Values are means ± SEM for n = 6.

Histamine release (% of total)

12-epi-scalaradial	[$sPLA_2$-II + IgE]	[$sPLA_2$-II] + [IgE]
0.1 μM	36.87 ± 5.10**	19.27 ± 4.23
0.3 μM	22.65 ± 4.66**	10.49 ± 3.09
0.6 μM	1.19 ± 0.82	0.36 ± 0.20
1.0 μM	1.71 ± 0.80	0.00 ± 0.00

[$sPLA_2$-II + IgE] = histamine release induced by anti-rat IgE from $sPLA_2$-II pretreated cells in the presence of 12-epi-scalaradial as observed in the experiment. [$sPLA_2$-II] + [IgE] = combined histamine release, calculated by adding the release induced in the presence of 12-epi-scalaradial by $sPLA_2$-II alone to that induced by anti-rat IgE alone for each individual experiment.

Table 3.15 (a + b) Comparison between (a) histamine release and (b) PGD_2 production, induced by anti-rat IgE from $sPLA_2$-II pretreated purified rat peritoneal mast cells with the calculated combined values from $sPLA_2$-II and anti-rat IgE. Anti-rat IgE induced histamine release was 27.33 ± 1.29% and PGD_2 production was 33.19 ± 6.73 ng/10^6 cells. ** = $p \leq 0.01$ and * = $p \leq 0.05$ as compared with the calculated combined value. Values are means ± SEM for n = 4-5.

Table 3.15 a **Histamine release (% of total)**

$sPLA_2$-II	[$sPLA_2$-II + IgE]	[$sPLA_2$-II] + [IgE]
1 Unit ml^{-1}	66.44 ± 2.13**	37.08 ± 1.23
0.1 Units ml^{-1}	55.14 ± 2.47**	30.25 ± 1.25
0.01 Units ml^{-1}	47.17 ± 3.01**	29.32 ± 0.89
0.001 Units ml^{-1}	36.68 ± 2.34**	28.64 ± 1.59

Table 3.15 b **PGD_2 production (ng/10^6 cells)**

$sPLA_2$-II	[$sPLA_2$-II + IgE]	[$sPLA_2$-II] + [IgE]
1 Unit ml^{-1}	164.19 ± 23.70*	74.50 ± 15.76
0.1 Units ml^{-1}	146.49 ± 34.82*	52.68 ± 11.49
0.01 Units ml^{-1}	124.86 ± 27.59	46.22 ± 7.32
0.001 Units ml^{-1}	71.86 ± 27.15	39.57 ± 7.44

[$sPLA_2$-II + IgE] = histamine release and PGD_2 production induced by anti-rat IgE from $sPLA_2$-II pretreated cells as observed in the experiment.

[$sPLA_2$-II] + [IgE] = combined histamine release and PGD_2 production, calculated by adding the release/production induced by $sPLA_2$-II alone to that induced by anti-rat IgE alone for each individual experiment.

3.6 Discussion

3.6.1 Effects of $sPLA_2$ on immunologically induced histamine release from activated RPMC

Pretreatment of rat peritoneal mast cells with the $sPLA_2$-I isolated from *Naja naja* venom followed by activation with anti-rat IgE led to a significant enhancement of the immunologically induced histamine release. These results are in agreement with Nagai *et al.* (1991) who observed that non-releasing concentrations of *Naja naja* venom (0.01 to 0.5 U ml^{-1}) accelerated antigen induced histamine release from rat peritoneal mast cells. Likewise, pretreatment of rat peritoneal mast cells with the $sPLA_2$-II purified from *Crotalus altrox* venom followed by immunological activation also led to a significant enhancement of the induced histamine release. This is in contrast with the findings reported by Murakamai *et al.* (1991a). They found that histamine release was unaffected in antigen stimulated rat peritoneal mast cells following pretreatment with the $sPLA_2$-II isolated from rat platelets (6 μg/ml). In the present study both $sPLA_2$ enzymes (0.4 to 40 μg/ml) significantly enhanced the anti-IgE induced histamine release dose dependently. This synergistic effect was observed with both the mixed cell population and purified cells. Concentrations of the $sPLA_2$ enzymes that caused enhancement in the present study, were similar to the concentrations found associated with acute pancreatitis. The normal serum $sPLA_2$ concentrations are 5.1 ng/ml (range 2.0 to 7.9 ng /ml) for healthy individuals and this was elevated in patients with acute pancreatitis (range 10 to 4,000 ng/ml) (Nishijima *et al.*, 1983). It was also observed that at low concentrations of the immunological stimulus, a significant enhancement

of histamine release was observed with both $sPLA_2$ enzymes. This would suggest that the $sPLA_2$ enzymes may have a role in priming the mast cell for degranulation.

The enhancement was unaffected by the cytosolic PLA_2 inhibitor MAFP, but was reduced with the PLA_2 inhibitors *p*-BPB and 12-epi-scalaradial. The enhancement was reduced with inhibitor concentrations that reduced the histamine releasing activity of these enzymes. The observations in the present study would suggest that the enzymatic site/activity of the $sPLA_2$ may be responsible for the effects observed here. However, a reduction in histamine release was also observed with the immunological stimulus anti-IgE and complicates these analyses. Murakamai *et al.* (1991b & 1992b) have also reported similar findings, where the PLA_2 inhibitors *p*-BPB, mepacrine, anti-(rat 14 kDa group II PLA_2) antibody, rat C3α and thielocin A1 all inhibited antigen induced histamine release from rat peritoneal mast cells. The 14 kDa group II PLA_2 inhibitors, rat C3α and thielocin A1 also inhibited histamine release induced by A23187 and compound 48/80 (Murakamai *et al.*, 1992b). They proposed that the type II $sPLA_2$ enzyme may play a central role in the activation/degranulation of mast cells.

Hydrolysis of the membrane phospholipids may generate a lipid substance which may be responsible for the enhanced histamine release. Bevers *et al.* (1983) reported that in activated platelets, PE and PS were translocated to the outer layer of the plasma membrane. Cross linking of the IgE receptor molecules on the mast cell membrane may lead to a membrane rearrangement event. This could make the specific phospholipid substrates PE and PS available for hydrolysis by $sPLA_2$-II.

PS at low concentrations (10 to 50 μg ml^{-1}) has been reported to enhance histamine release from rat peritoneal mast cells induced by dextran, concanavalin A (con A), antigen or anti-IgE while it was without effect by itself (Goth *et al.*, 1971; Baxter & Adamik, 1977). This enhancement was specific for agents which activate mast cells through the immunological pathway, as it had no enhancing effect on other mast cell secretatogues such as compound 48/80 and A23187. The other phospholipids phosphatidylinositol (PI), PE and PC had no enhancing effect. Hydrolysis of PS by PLA_2 yields lysoPS and this lyso derivative at low concentrations (less than 10 μM) has also been shown to enhance histamine release from rat peritoneal mast cells activated with con A (Martin & Lagunoff, 1979; Smith *et al.*, 1979; Boarato *et al.*, 1984; Horigome *et al.*, 1986). LysoPS is about 50 to 1,000 times more potent than PS and was without effect on resting rat peritoneal mast cells. Higher concentrations of lysophospholipids are known to induce cytotoxic histamine release from rat peritoneal mast cells (Martin & Lagunoff, 1979). Hydrolysis of PS by $sPLA_2$-II could generate lysoPS. This lysoPS may in some way be responsible for the enhanced histamine release observed here.

$sPLA_2$-I does not display any substrate selectivity for PS. However, a significant enhancement was observed with non-releasing concentrations of $sPLA_2$-I. $sPLA_2$-I may bind to a binding site which facilitates the release of histamine from mast cells and release of the $sPLA_2$ found in mast cell granules. Rat peritoneal mast cells release a 14kDa type II PLA_2 when stimulated with antigen (Murakami *et al.*, 1992a). More recently this 14kDa PLA_2 has been located in the secretory granules of rat peritoneal mast cells (Chock *et al.*, 1994). This secreted type II 14kDa $sPLA_2$ could hydrolyse

PS to yield lysoPS or another fusogenic compound and in this way augment the induced histamine release. Release of this 14kDa type II $sPLA_2$ following cell activation may explain the enhanced histamine release observed with $sPLA_2$-I in this study. Significant enhancement was observed with the longer activation time with anti-IgE, suggesting that time may be needed for secretion of the 14kDa type II $sPLA_2$ from the mast cell granules.

The function of the type II $sPLA_2$ secreted from mast cell granules following cell activation is unclear. The observations in the present study suggest that it augments immunologically induced histamine release. It may mediate this effect by acting as a signalling molecule through binding a site on the mast cell plasma membrane. Cross linking of the IgE receptor molecules on the mast cell membrane may lead to a membrane alteration event which may expose a specific binding site for $sPLA_2$-II. However, no such binding site has been isolated on mast cells to date. The type II $sPLA_2$ displays a high affinity for heparin (Chock *et al.*, 1991; Murakami *et al.*, 1992a). This affinity for heparin may help anchor the enzyme on the cell surface in order to mediate its effect. Alternatively, $sPLA_2$-II may also bind with low affinity to the type I $sPLA_2$-I binding site and mediate its effect that way. Hydrolysis of PS by $sPLA_2$-II would generate lysoPS and this lysoPS may in some way be responsible for the enhanced histamine release observed here. More studies are required to establish the mechanisn of action of $sPLA_2$-II involved in enhancing histamine release from rat peritoneal mast cells.

3.6.2 Effects of $sPLA_2$ on PGD_2 production from immunologically activated RPMC

PGD_2 production was enhanced in anti-IgE stimulated mast cells preincubated with the type II $sPLA_2$ enzyme purified from *Crotalus altrox* venom. These results are in aggreement with Murakami *et al.* (1991a). They observed that preincubation of rat peritoneal mast cells with the the type II 14kDa $sPLA_2$ (6 $\mu g\ ml^{-1}$) isolated from rat platelets, subsequently activated with antigen, led to an increase in PGD_2 production. Hara *et al.* (1991) also observed that in A23187 activated HL-60 granulocytes PGE_2 production was increased in the presence of exogenous rat and human type II PLA_2. Foneth *et al.* (1994) reported that the mouse mast cell line (BBMC) secrete a 14kDa $sPLA_2$ following antigen challenge. This $sPLA_2$-II enzyme leads to the rapid mobilisation of extracellular AA which is subsequently used for eicosanoid generation. The enhanced PGD_2 production observed here is probably due to the release of more AA from the membrane phospholipid, following the enzymatic action of the secreted PLA_2-II.

In contrast with $sPLA_2$-II, there was no significant enhancement of PGD_2 production observed with $sPLA_2$-I following activation with anti-rat IgE. $sPLA_2$-I may bind to a binding site on the mast cell plasma membrane and activate histamine secretion. In addition, this would lead to the release of the 14kDa $sPLA_2$ found in the mast cell granules. Time may be required for its secretion and action on liberating free AA for PGD_2 production. The lack of enhancement observed here may be due to this time factor.

3.6.3 Effects of flurbiprofen and zileuton on $sPLA_2$ induced and enhanced histamine release from RPMC

Both the cyclo-oxygenase (flurbiprofen) and lipoxygenase (zileuton) inhibitors failed to inhibit the $sPLA_2$ induced histamine release and the enhanced immunologically induced histamine release. These results are in accord with Nagai *et al.* (1991) who observed that indomethacin and AA-861, were without effect on the porcine pancreas and the $sPLA_2$ isolated from *Naja naja* venom enhanced antigen induced histamine release from rat peritoneal mast cells. This suggested that the AA metabolites generated through the cyclo-oxygenase and lipoxygenase pathway were not responsible for the effects of the $sPLA_2$ enzymes in the present study. These results further support the hypothesis that the $sPLA_2$ enzymes were having a direct effect on the mast cell plasma membrane either through binding sites or a hydrolytic effect.

The present study has shown that both $sPLA_2$ enzymes enhanced immunologically induced histamine release and this was not do to the generation of AA metabolites, which suggested that the $sPLA_2$ enzymes may participate in some step required for anti-IgE induced release. If the $sPLA_2$ enzyme was involved in a common step in the secretory process, an enhancement of histamine release induced by other mast cell secretagogues such as compound 48/80 or A23187 would also be expected. Although the mechanisms by which mast cell secretagogues cause exocytosis are not fully understood, they differ in their requirement for extracellular calcium (Pearce, 1987). The immunological activation of mast cells is highly dependent on the calcium concentration in the extracellular medium, the release induced by compound 48/80 is

much less sensitive (Pearce *et al.*, 1981). The calcium ionophore A23187 is also dependent on the calcium concentration in the extracellular medium, but can bypass all membrane activation events thus raising the $[Ca^{2+}]_i$ within the cell cytosol (Foreman *et al.*, 1973). The effects of the $sPLA_2$ enzymes on non-immunologically induced histamine release were next examined and their effects on intracellular calcium measurements were also investigated (Part 3).

PART 3: Investigation of the mode of action(s) of $sPLA_2$ on rat peritoneal mast cells

3.7 Results

3.7.1 Effects of $sPLA_2$-I on non-immunologically activated RPMC

Mast cells can also be activated with non-immunological stimuli. The calcium ionophore A23187 and the classical mast cell degranulating agent compound 48/80 were used in this study. When $sPLA_2$-I preincubated cells were subsequently challenged with compound 48/80, there was no significant enhancement of compound 48/80 induced histamine release observed (Fig 3.32 and table 3.16). Concentrations of $sPLA_2$-I greater than 1 U ml^{-1} caused significant histamine release alone. Similarly there was no significant enhancement of histamine release observed when $sPLA_2$-I preincubated cells were activated with A23187 (Fig 3.33 and table 3.17).

3.7.2 Effects of $sPLA_2$-II on non-immunologically activated RPMC

It was also observed that $sPLA_2$-II preincubated cells did not produce any significant enhancement of compound 48/80 induced histamine release (Fig 3.34 and table 3.18). At the highest concentration tested of 10 U ml^{-1}, there was a reduction in the histamine release induced by compound 48/80. The histamine release observed was 12.02 $\pm$ 2.47% compared with 26.02 $\pm$ 3.65% (compound 48/80 alone). Similar observations were seen when $sPLA_2$-II preincubated cells were challenged with A23187 (Fig 3.35).

There was no enhanced histamine release observed (Table 3.19). Again an $sPLA_2$-II concentration of 10 U ml^{-1} reduced the A23187 induced histamine release by one fold.

3.7.3 Effects of $sPLA_2$-I on $[Ca^{2+}]_i$ in immunologically activated RPMC

When purified rat peritoneal mast cells, loaded with fura-2 were exposed to $sPLA_2$-I (0.1 U ml^{-1}) an increase in the $[Ca^{2+}]_i$ was observed (Fig 3.36a). This increase was slow and reached a plateau within 2 minutes. The net increase in the $[Ca^{2+}]_i$ observed was 23.66 $\pm$ 6.00 nM for n = 5. The corresponding % histamine release observed was 2.70 $\pm$ 1.30%.

When fura-2 loaded cells were exposed to anti-rat IgE (1/3,000) a biphasic calcium response was observed (Fig 3.36b). The initial peak was fast with a transient rise in the $[Ca^{2+}]_i$, followed by a sustained plateau phase. The net increase in the $[Ca^{2+}]_i$ observed was 78.89 $\pm$ 3.83 nM for the peak and 53.30 $\pm$ 9.63 nM for the plateau phase. The corresponding % histamine release observed was 6.61 $\pm$ 0.33%.

To determine whether $sPLA_2$-I was having any effect on the anti-rat IgE induced calcium response, cells were exposed to $sPLA_2$-I for 5 min before the addition of anti-IgE. Fig 3.36c illustrates that exposure to $sPLA_2$-I led to a slow increase in the $[Ca^{2+}]_i$ as before. Upon addition of anti-IgE the rapid transient increase in the $[Ca^{2+}]_i$ was again observed. This was followed by the sustained phase. The net increase in the $[Ca^{2+}]_i$ observed was 22.86 $\pm$ 2.86 nM for $sPLA_2$-I, 94.17 $\pm$ 10.89 nM for the peak and 82.82 $\pm$ 13.71 nM for the plateau phase. The corresponding % histamine release

observed was 13.00 ± 1.20%.

The increase in the $[Ca^{2+}]_i$ by $sPLA_2$-I complicates these analyses. However, subtraction of this net increase in the $[Ca^{2+}]_i$ by $sPLA_2$-I from the experimental observations above showed that $sPLA_2$-I did not have any significant effect on the anti-rat IgE induced calcium response. The % histamine release observed for these traces was less than 15%. It should also be noted that in these experiments there was no significant enhancement of the anti-IgE induced histamine release from $sPLA_2$-I pretreated cells (13.00 ± 1.20%) when compared with the calculated combined value (9.32 ± 1.38%).

3.7.4 Effect of $sPLA_2$-II on $[Ca^{2+}]_i$ in immunologically activated RPMC

When mast cells, loaded with fura-2 were exposed to $sPLA_2$-II (0.1 U ml^{-1}), an increase in the $[Ca^{2+}]_i$ was observed (Fig 3.37a). This increase was slow and reached a plateau within 2 minutes. The net increase in the $[Ca^{2+}]_i$ observed was 11.20 ± 2.57 nM for n = 6 compared with 23.66 ± 6.00 nM for $sPLA_2$-I (0.1 U ml^{-1}). The corresponding % histamine release observed was 3.92 ± 1.14%.

As before when fura-2 loaded cells were exposed to anti-rat IgE (1/3,000), the biphasic calcium response was observed (Fig 3.37b). The net increase in the $[Ca^{2+}]_i$ observed was 87.11 ± 10.30 nM for the peak and for the plateau phase 51.07 ± 5.80 nM. The corresponding % histamine release observed was 13.05 ± 2.07%.

To determine whether $sPLA_2$-II was having any effect on the anti-rat IgE calcium response, cells were exposed to $sPLA_2$-II for 5 min before the addition of anti-IgE. Fig 3.37c illustrates that exposure to $sPLA_2$-II led to a slow increase in the $[Ca^{2+}]_i$ as before. Upon addition of anti-rat IgE a rapid transient increase in the $[Ca^{2+}]_i$ was again observed which was followed by the sustained phase. The net increase in the $[Ca^{2+}]_i$ observed was 11.91 ± 2.35 nM for $sPLA_2$-II, 102.12 ± 10.89 nM for the peak and for the plateau phase 87.12 ± 10.30 nM. The corresponding % histamine release observed was $27.61 \pm 2.21\%$.

The increase in the $[Ca^{2+}]_i$ by $sPLA_2$-II complicates these analyses. Subtraction of this net increase in the $[Ca^{2+}]_i$ by $sPLA_2$-II from the experimental observations above showed that $sPLA_2$-II did have a significant effect on the anti-IgE induced calcium response. However it was only with the plateau phase that there was a significant increase observed in the $[Ca^{2+}]_i$. It should also be noted that there was a significant enhancement of the anti-rat IgE induced histamine release from $sPLA_2$-II pretreated cells ($27.61 \pm 2.21\%$) when compared with the calculated combined value ($16.98 \pm 2.78\%$). This corresponds with the increased $[Ca^{2+}]_i$ following cell activation.

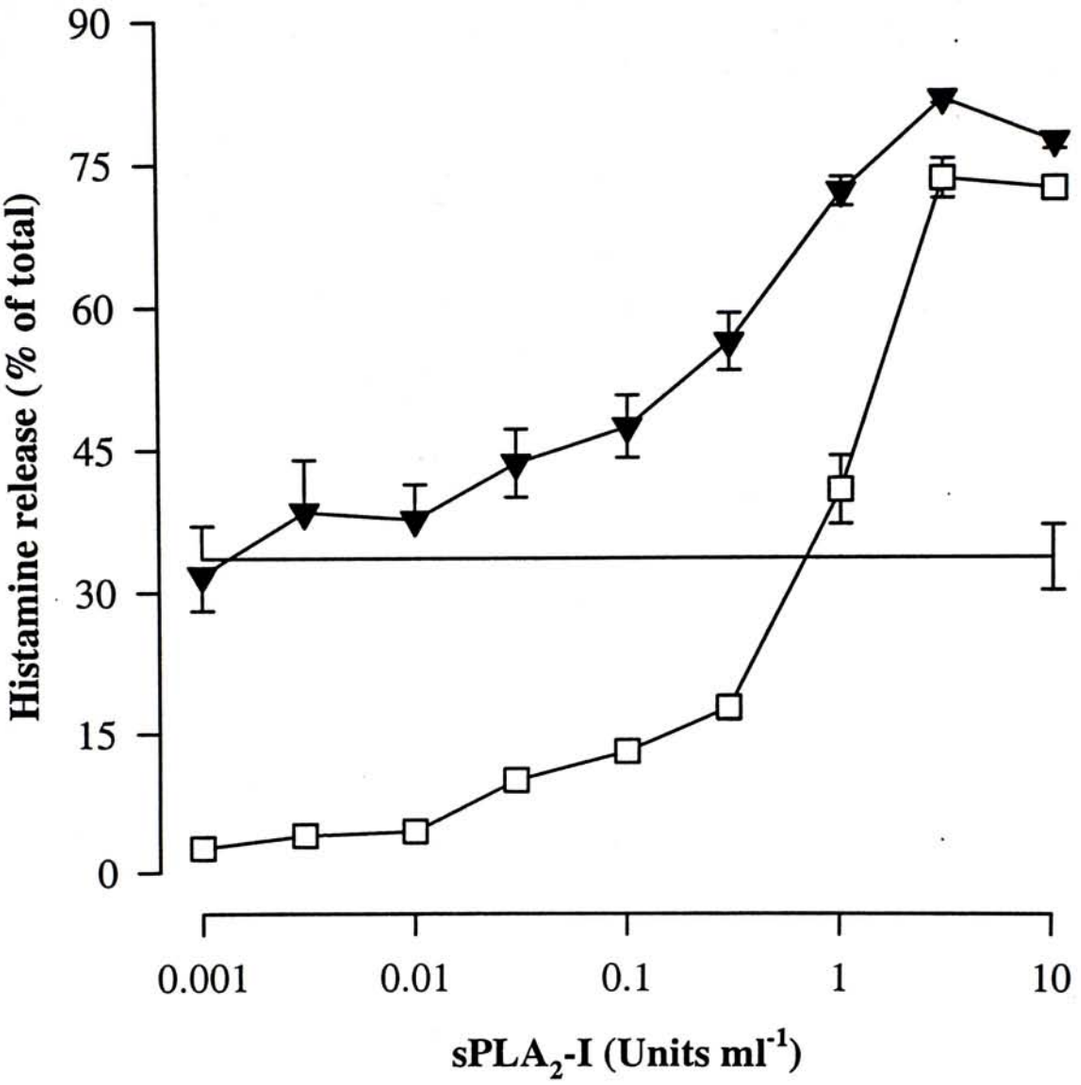

Figure 3.32 Effects of $sPLA_2$-I on compound 48/80 induced histamine secretion from purified rat peritoneal mast cells. Control cells were incubated with $sPLA_2$-I (0.001 to 10 U ml^{-1}) for 20 min (□). Compound 48/80 stimulated cells were pretreated with $sPLA_2$-I for 10 min and stimulated for a further 10 min (▼). Compound 48/80 (0.075 μg ml^{-1}) induced histamine release was 33.56 ± 3.47% (—). The spontaneous histamine release was 9.59 ± 0.63%. Results are given as the means ± SEM for n = 6-7.

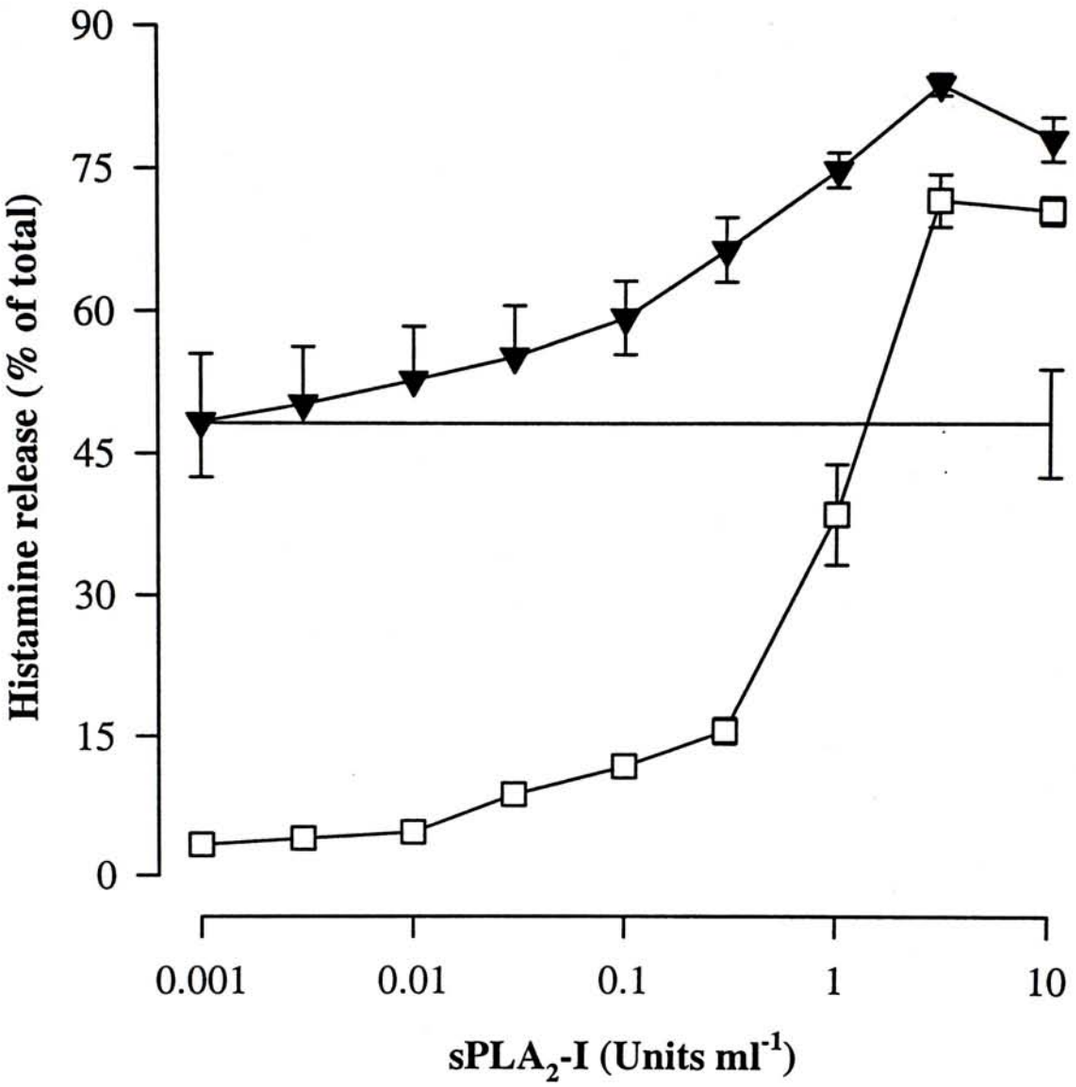

Figure 3.33 Effects of $sPLA_2$-I on A23187 induced histamine secretion from purified rat peritoneal mast cells. Control cells were incubated with $sPLA_2$-I (0.001 to 10 U ml^{-1}) for 20 min (□). A23187 stimulated cells were pretreated with $sPLA_2$-I for 10 min and stimulated for a further 10 min (▼). A23187 (0.5 μM) induced histamine release was 48.21 ± 5.73% (—). The spontaneous histamine release was 10.48 ± 0.62%. Results are given as the means ± SEM for n = 5.

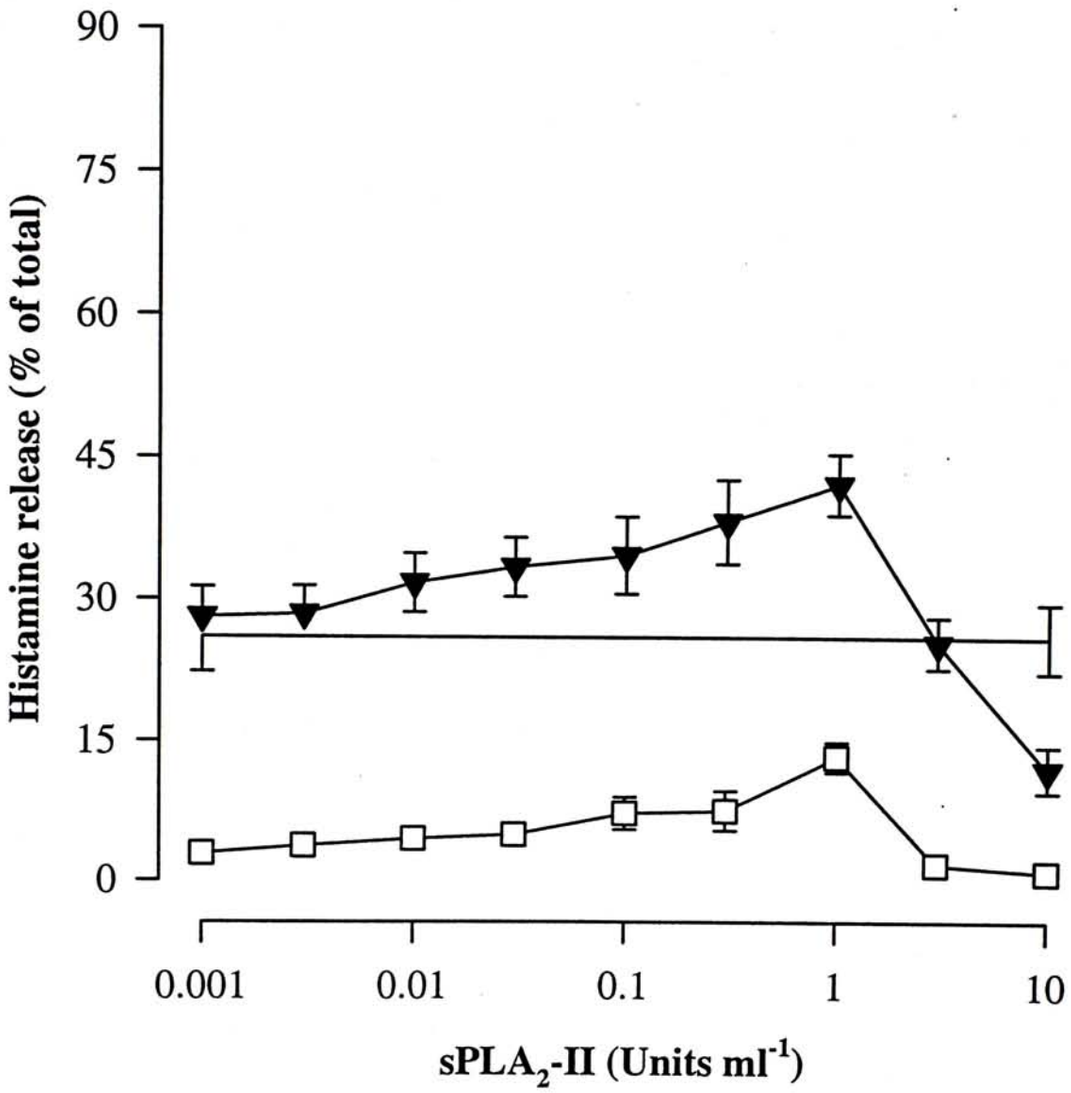

Figure 3.34 Effects of $sPLA_2$-II on compound 48/80 induced histamine secretion from purified rat peritoneal mast cells. Control cells were incubated with $sPLA_2$-II (0.001 to 10 U ml^{-1}) for 20 min (□). Compound 48/80 stimulated cells were pretreated with $sPLA_2$-II for 10 min and stimulated for a further 10 min (▼). Compound 48/80 (0.075 μg ml^{-1}) induced histamine release was 26.02 ± 3.65% (—). The spontaneous histamine release was 12.59 ± 1.77%. Results are given as the means ± SEM for n = 3-5.

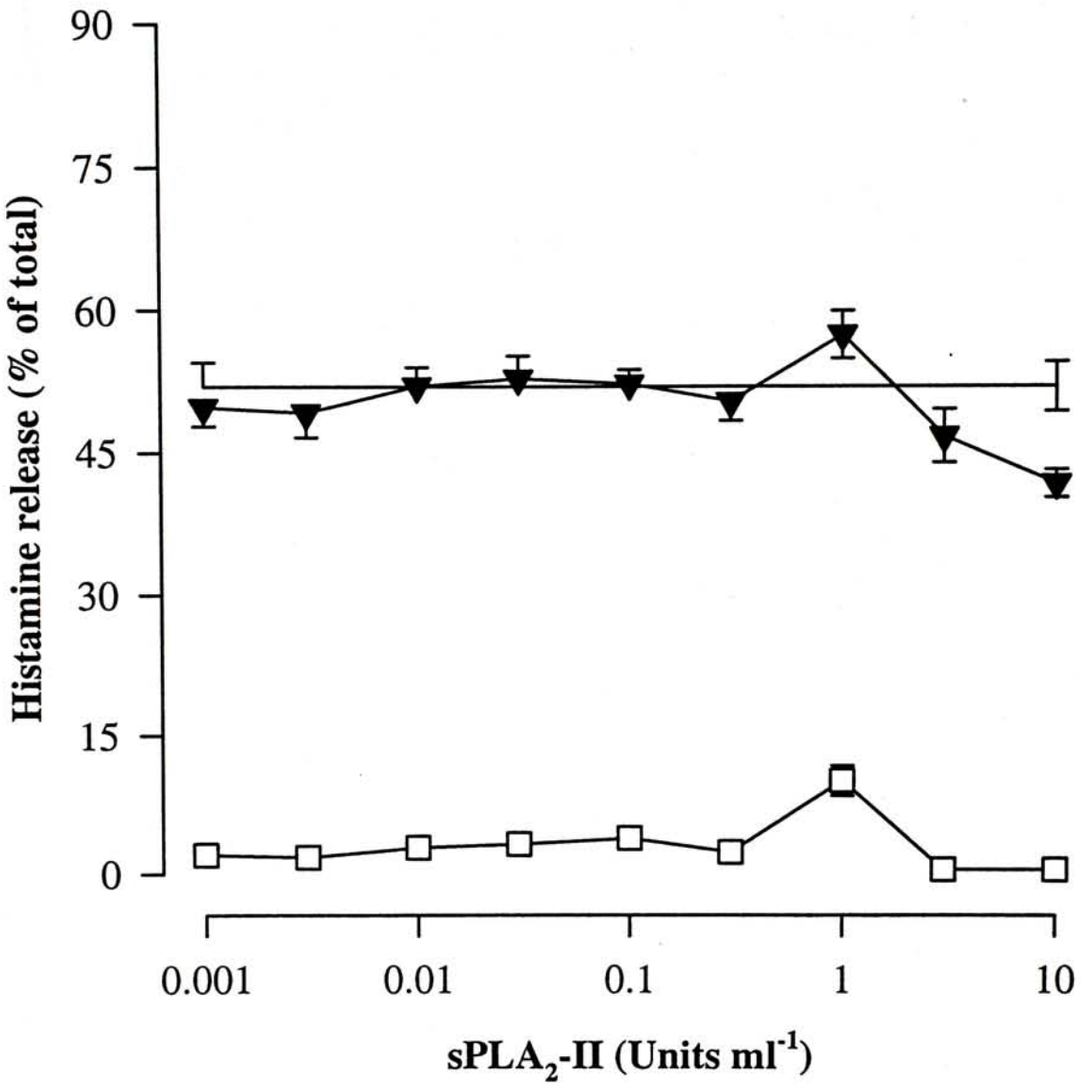

Figure 3.35 Effects of $sPLA_2$-II on A23187 induced histamine secretion from purified rat peritoneal mast cells. Control cells were incubated with $sPLA_2$-II (0.001 to 10 U ml^{-1}) for 20 min (□). A23187 stimulated cells were pretreated with $sPLA_2$-I for 10 min and stimulated for a further 10 min (▼). A23187 (0.5 μM) induced histamine release was 51.93 ± 2.61% (—). The spontaneous histamine release was 12.49 ± 1.58%. Results are given as the means ± SEM for n = 3.

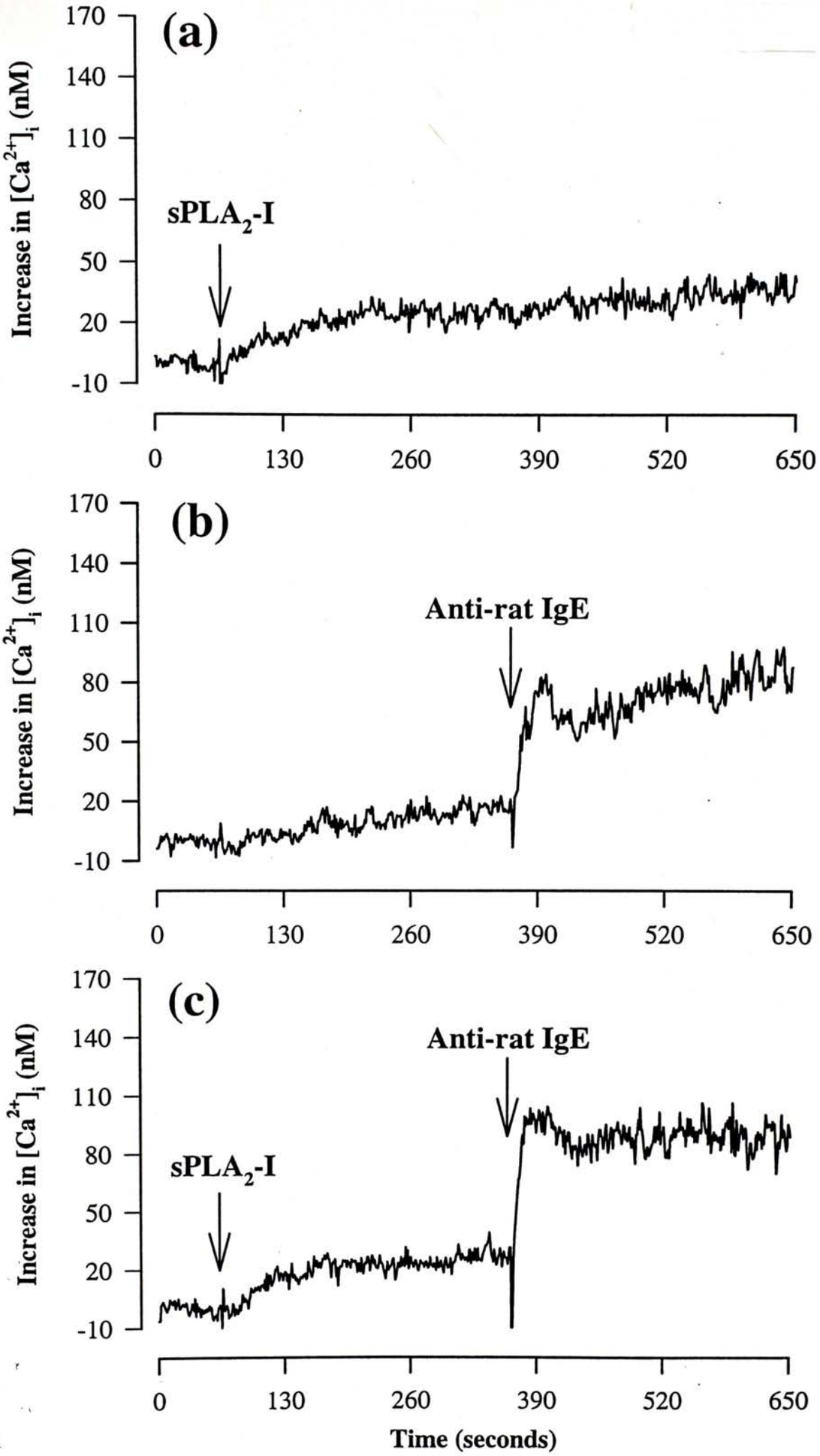

Figure 3.36 Effects of (a) $sPLA_2$-I (0.1 U ml^{-1}), (b) anti-rat IgE (1/3,000) and (c) $sPLA_2$-I + anti-rat IgE, on $[Ca^{2+}]_i$ in fura-2 loaded purified rat peritoneal mast cells in Hepes buffer. Traces are shown for changes in $[Ca^{2+}]_i$ representative for n = 5.

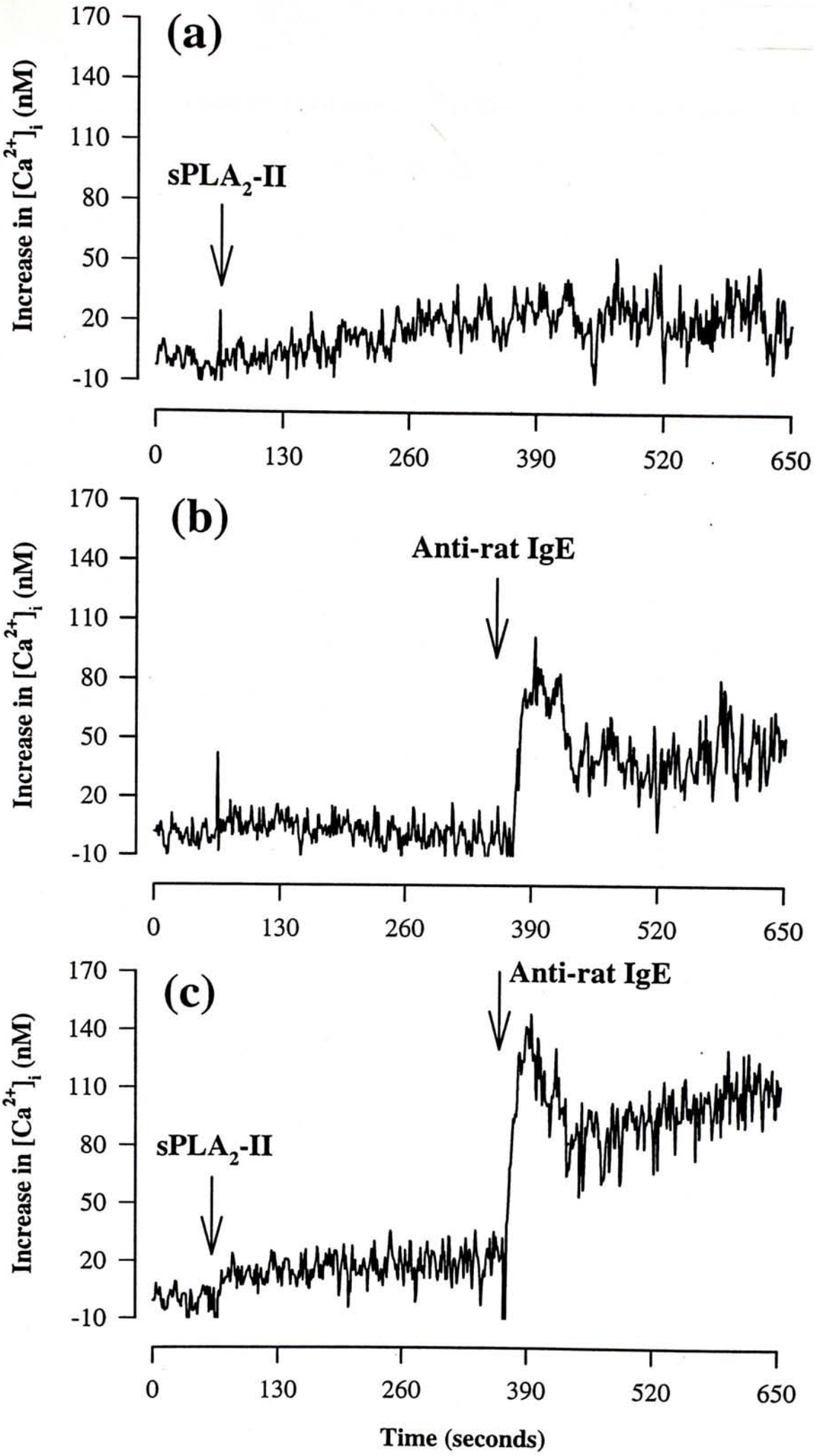

Figure 3.37 Effects of (a) $sPLA_2$-II (0.1 U ml^{-1}), (b) anti-rat IgE (1/3,000) and (c) $sPLA_2$-II + anti-rat IgE, on $[Ca^{2+}]_i$ in fura-2 loaded purified rat peritoneal mast cells in Hepes buffer. Traces are shown for changes in $[Ca^{2+}]_i$ representative for n = 6.

Table 3.16 Comparison between histamine release induced by compound 48/80 from $sPLA_2$-I pretreated purified rat peritoneal mast cells with the calculated combined histamine release induced by $sPLA_2$-I and compound 48/80. Compound 48/80 (0.075 $\mu g\ ml^{-1}$), induced histamine release was 33.56 ± 3.47%. Values are means ± SEM for n = 6-7.

	Histamine release (% of total)	
$sPLA_2$-I	**[$sPLA_2$-I + 48/80]**	**[$sPLA_2$-I] + [48/80]**
10 Units ml^{-1}	77.38 ± 0.85	105.94 ± 3.21
3 Units ml^{-1}	81.95 ± 0.52	107.08 ± 3.43
1.0 Unit ml^{-1}	72.20 ± 1.53	74.31 ± 4.33
0.3 Units ml^{-1}	56.42 ± 3.02	51.25 ± 3.19
0.1 Units ml^{-1}	47.47 ± 3.28	46.59 ± 3.21
0.03 Units ml^{-1}	43.64 ± 3.60	43.47 ± 3.69
0.01 Units ml^{-1}	37.66 ± 3.72	37.98 ± 3.87
0.003 Units ml^{-1}	38.47 ± 5.51	37.50 ± 3.98
0.001 Units ml^{-1}	31.86 ± 3.80	35.73 ± 4.68

[$sPLA_2$-I + 48/80] = histamine release induced by compound 48/80 from $sPLA_2$-I pretreated cells as observed in the experiment.

[$sPLA_2$-I] + [48/80] = combined histamine release, calculated by adding the release induced by $sPLA_2$-I alone to that induced by compound 48/80 alone for each individual experiment.

Table 3.17 Comparison between histamine release induced by A23187 from $sPLA_2$-I pretreated purified rat peritoneal mast cells with the calculated combined histamine release induced by $sPLA_2$-I and A23187. A23187 (0.5 μM), induced histamine release was 48.21 ± 5.73%. Values are means ± SEM for n = 5.

	Histamine release (% of total)	
$sPLA_2$-I	**[$sPLA_2$-I + A23187]**	**[$sPLA_2$-I] + [A23187]**
10 Units ml^{-1}	78.31 ± 2.30	118.89 ± 6.26
3 Units ml^{-1}	84.01 ± 1.16	119.97 ± 6.76
1.0 Unit ml^{-1}	74.97 ± 1.86	86.77 ± 8.58
0.3 Units ml^{-1}	66.55 ± 3.39	63.77 ± 6.40
0.1 Units ml^{-1}	59.40 ± 3.89	60.02 ± 5.52
0.03 Units ml^{-1}	55.30 ± 5.34	56.98 ± 5.50
0.01 Units ml^{-1}	52.81 ± 5.60	52.88 ± 5.44
0.003 Units ml^{-1}	50.28 ± 5.97	52.22 ± 4.88
0.001 Units ml^{-1}	48.39 ± 7.11	51.57 ± 4.90

[$sPLA_2$-I + A23187] = histamine release induced by A23187 from $sPLA_2$-I pretreated cells as observed in the experiment.

[$sPLA_2$-I] + [A23187] = combined histamine release, calculated by adding the release induced by $sPLA_2$-I alone to that induced by A23187 alone for each individual experiment.

Table 3.18 Comparison between histamine release induced by compound 48/80 from $sPLA_2$-II pretreated purified rat peritoneal mast cells with the calculated combined histamine release induced by $sPLA_2$-II and compound 48/80. Compound 48/80 (0.075 $\mu g\ ml^{-1}$), induced histamine release was 26.02 ± 3.65%. ** = $p \leq 0.01$ as compared with the calculated combined value. Values are means ± SEM for n = 3-5.

Histamine release (% of total)

$sPLA_2$-II	**[$sPLA_2$-II + 48/80]**	**[$sPLA_2$-II] + [48/80]**
10 Units ml^{-1}	12.02 ± 2.47**	26.87 ± 3.94
3 Units ml^{-1}	25.49 ± 2.71	27.69 ± 3.79
1.0 Unit ml^{-1}	42.31 ± 3.23	39.35 ± 4.90
0.3 Units ml^{-1}	38.32 ± 4.44	33.52 ± 4.96
0.1 Units ml^{-1}	34.74 ± 4.10	33.25 ± 5.20
0.03 Units ml^{-1}	33.49 ± 3.14	30.94 ± 4.62
0.01 Units ml^{-1}	31.80 ± 3.11	30.18 ± 4.70
0.003 Units ml^{-1}	28.48 ± 2.95	30.04 ± 6.17
0.001 Units ml^{-1}	28.13 ± 3.20	31.40 ± 7.58

[$sPLA_2$-II + 48/80] = histamine release induced by compound 48/80 from $sPLA_2$-II pretreated cells as observed in the experiment.

[$sPLA_2$-II] + [48/80] = combined histamine release, calculated by adding the release induced by $sPLA_2$-II alone to that induced by compound 48/80 alone for each individual experiment.

Table 3.19 Comparison between histamine release induced by A23187 from $sPLA_2$-II pretreated purified rat peritoneal mast cells with the calculated combined histamine release induced by $sPLA_2$-II and A23187. A23187 (0.5 μM), induced histamine release was 51.93 ± 2.61%. * = $p \leq 0.05$ as compared with the calculated combined value. Values are means ± SEM for n = 3.

Histamine release (% of total)

$sPLA_2$-II	**[$sPLA_2$-II + A23187]**	**[$sPLA_2$-II] + [A23187]**
10 Units ml^{-1}	41.70 ± 1.50*	52.40 ± 2.16
3 Units ml^{-1}	46.73 ± 2.82	52.41 ± 2.14
1.0 Unit ml^{-1}	57.41 ± 2.49	61.96 ± 1.47
0.3 Units ml^{-1}	50.36 ± 1.99	54.29 ± 2.13
0.1 Units ml^{-1}	52.26 ± 1.47	55.78 ± 2.47
0.03 Units ml^{-1}	52.82 ± 2.40	55.17 ± 2.63
0.01 Units ml^{-1}	52.01 ± 1.99	54.75 ± 1.71
0.003 Units ml^{-1}	49.20 ± 2.58	53.72 ± 1.94
0.001 Units ml^{-1}	49.77 ± 2.00	54.00 ± 2.32

[$sPLA_2$-II + A23187] = histamine release induced by A23187 from $sPLA_2$-II pretreated cells as observed in the experiment.

[$sPLA_2$-II] + [A23187] = combined histamine release, calculated by adding the release induced by $sPLA_2$-II alone to that induced by A23187 alone for each individual experiment.

3.8 Discussion

The specificity of the $sPLA_2$ enzymes to enhance immunologically induced histamine release, suggested that they may participate in some step specifically required for IgE induced release. Although the exact mechanisms involved in mast cell secretion are not fully understood, it should be pointed out that they differ in their requirement for extracellular calcium (Pearce, 1987). While the immunological activation of mast cells is highly dependent on the calcium concentration in the extracellular medium, the release induced by compound 48/80 is much less sensitive (Pearce *et al.*, 1981). The calcium ionophore A23187 is also dependent on the calcium concentration in the extracellular medium, but can bypass all membrane activation events thus raising the $[Ca^{2+}]_i$ within the cell cytosol (Foreman *et al.*, 1973).

The sequence of events following aggregation of the IgE receptor molecules on the mast cell plasma membrane have not yet been fully determined. It is known that this aggregation step leads to the intracellular production of IP_3 and an increase in the $[Ca^{2+}]_i$. This increase in the $[Ca^{2+}]_i$ is due to the release of Ca^{2+} from intracellular stores by IP_3. The transient increase in the $[Ca^{2+}]_i$, is insufficient to initiate complete secretion, but leads to the opening of plasma membrane calcium channels. Entry of Ca^{2+} from the extracellular medium maintains an elevated level of the $[Ca^{2+}]_i$, which then facilitates the maximum level of mediator release (for review see Sagi-Eisenberg, 1993). This biphasic calcium response was observed in this study, when cells were stimulated with anti-rat IgE.

With compound 48/80 the first known intracellular event is thought to be the activation of a pertussis-toxin-sensitive G protein. This leads to the activation of PLC with an increase in IP_3. Although the optimal level of histamine release requires the presence of extracellular calcium, compound 48/80 can produce a significant release of histamine in the absence of extracellular calcium (Pearce *et al.*, 1981; for review see Sagi-Eisenberg, 1993). Hence the opening of plasma membrane calcium channels, is not a prerequisite for histamine release induced by compound 48/80.

The specificity of the $sPLA_2$ enzymes to enhance immunologically induced histamine release here, suggested that they may in some way facilitate in the opening, or maintenance of the opening of the plasma membrane calcium channels. Two calcium influx pathways have been described in mast cells: I_{CRAC} (calcium release-activated calcium) and a non specific 50-pS cation channel. I_{CRAC} has been characterised in rat peritoneal mast cells (Hoth & Penner, 1992 & 1993) and the RBL-2H3 cell line (Fasolato *et al.*, 1993b; Zhang & McCloskey, 1995). The 50-pS channel has also been demonstrated in rat peritoneal mast cells and is activated via G-proteins (Penner *et al.*, 1988; Matthews *et al.*, 1989; Kudo & Kimura, 1992; Fasolato *et al.*, 1993a). In this study only $sPLA_2$-II produced any significant increase in the influx of extracellular Ca^{2+} following stimulation with anti-IgE. This influx in Ca^{2+} corresponded with the observed enhanced histamine release. This suggested that $sPLA_2$-II may facilitate in the opening of plasma membrane calcium channels. The mechanism(s) of action of how $sPLA_2$-II might do this is not clear at present.

As previously discussed, both PS and lysoPS can enhance immunologically induced

histamine release, but not that of compound 48/80 or A23187. It has been reported that PS can enhance antigen induced ^{45}Ca uptake in rat peritoneal mast cells (Foreman *et al.*, 1977; Ishizaka *et al.*, 1979). Lloret & Moreno (1995) also reported that LysoPS could induce a significant ^{45}Ca influx in mouse mast cells. The lysoPS-induced histamine release was blocked by apomorphine which inhibits cyclic nucleotide phosphodiesterase and EDTA, further supporting a role for Ca^{2+}. These authors proposed that the histamine release induced by lysoPS was mediated by a GTP-binding protein and subsequent PLC activation. Pertussis toxin blocked the histamine releasing activity. Secondly lysoPS also stimulated inositol phosphate formation. These reports would support our observations with the increased Ca^{2+} influx observed here with $sPLA_2$-II following stimulation with anti-IgE. However, the mechanism of action is unclear and further experiments are needed.

There was no enhancement of the Ca^{2+} signal observed when cells preincubated with $sPLA_2$-I, were stimulated with anti-rat IgE. There was also no corresponding enhanced histamine release observed. This could be due to the time factor, since cells were preincubated with $sPLA_2$-I for 5 min before activation with anti-rat IgE for a further 5 min for calcium measurements. A significant enhancement of histamine release was only observed with a 10 min preincubation time with $sPLA_2$-I and a 10 min activation time with anti-rat IgE. The shorter incubation times were employed here to reduce the problem of dye leakage. In addition, in this study with a 20 min reaction time, cell clumping was observed. This may represent the death of some cells due to a poor oxygen supply since a high number of cells were present in the cuvette in a small volume of buffer. The spontaneous calcium measurement also increased with

the reaction time of 20 min. $sPLA_2$-I may bind to a binding site on the mast cell plasma membrane and cause histamine release and release of the 14kDa type II $sPLA_2$ located in the secretory granules of mast cells. Thus time may be needed for its secretion and action on increasing the influx of calcium. This may explain why there was no significant increase observed with the Ca^{2+} signal.

Both $sPLA_2$ enzymes led to a slow increase in the $[Ca^{2+}]_i$. However, the net increase in the $[Ca^{2+}]_i$ observed with $sPLA_2$-I was 23.66 $\pm$ 6.00 nM compared with 11.20 $\pm$ 2.57 nM for $sPLA_2$-II (0.1 U ml^{-1}). This influx of extracellular calcium could be mediated by the opening of a calcium channel on the plasma membrane for the type II $sPLA_2$. With the type I $sPLA_2$ the calcium influx observed maybe due to its enzymatic activity since no potentiation was observed following activation with anti-IgE. Binding of the $sPLA_2$ enzymes to a putative binding site could lead to an intracellular signalling event which could activate the opening of a calcium channel in the plasma membrane. Alternatively, the $sPLA_2$ enzymes may disrupt the plasma membrane through their enzymatic activity and this could lead to the observed calcium influx.

3.9 Summary of the possible mechanisms of action of the $sPLA_2$ enzymes

The physiological function of the $sPLA_2$ enzymes originally isolated from the pancreatic juices were of a digestive nature. Within the past decade, the identification of $sPLA_2$ enzymes in tissues and in inflammatory conditions has questioned the function of these $sPLA_2$ enzymes. More recently, the identification of specific receptors for these $sPLA_2$ enzymes on cells and tissues has suggested that they may function as signalling molecules. In this study two main hypotheses: (a) catalytic action and (b) signalling molecule, were proposed to explain the effects observed with the type I $sPLA_2$ isolated from *Naja naja* venom and the type II $sPLA_2$ isolated from *Crotalus altrox* venom on resting and activated rat peritoneal mast cells.

Histamine release was observed with high concentrations of $sPLA_2$-I (0.5 to 10 U ml^{-1}) on resting mast cells. This histamine release required a metabolically active cell, extracellular calcium and the native structure of $sPLA_2$-I. $sPLA_2$-I could mediate its effects on unstimulated mast cells by directly digesting the mast cell plasma membrane. Hydrolysis of the plasma membrane phospholipid could disrupt the plasma membrane structure. This altered membrane structure could then allow the influx of extracellular ions such as calcium into the cell cytosol. A slow increase in the $[Ca^{2+}]_i$ was observed when fura-2 loaded cells were exposed to $sPLA_2$-I. This influx of calcium would be sufficient to cause histamine secretion since calcium plays a central role in mast cell degranulation (Pearce, 1985).

Bevers *et al.* (1983) reported that the outer layer of the plasma membrane in resting

platelets is occupied with PC. The type I $sPLA_2$ enzymes display a substrate specificity for PC or PE (Verheiji *et al.*, 1981). If the mast cell plasma membrane has a similar phospholipid composition like platelets, $sPLA_2$-I could hydrolyse this PC. Hydrolysis of PC could yield a lipid substance which could augment fusion of the mast cell plasma membrane with the secretory granules during histamine secretion. LysoPC was reported to have fusogenic properties (Poole *et al.*, 1970) but in mast cells lysoPC has a cytotoxic (Martin & Lagunoff, 1979) and inhibitory effect (Chernomordik *et al.*, 1993).

Alternatively, $sPLA_2$-I could bind to a putative binding site on the mast cell plasma membrane and act as a signalling molecule. Receptor sites for $sPLA_2$-I have been identified in a variety of cells and tissues, such as Swiss 3T3 fibroblast cells (Arita *et al.*, 1991), rabbit skeletal muscle (Lambeau *et al.*, 1990), and rat vascular smooth muscle (Hanasaki & Arita, 1992). The type I $sPLA_2$ receptors have also been cloned in rabbit (Lambeau *et al.*, 1994), bovine (Ishizaki *et al.*, 1994) and human (Ancian *et al.*, 1995) species. Binding of $sPLA_2$-I to a binding site, could activate an intracellular signalling pathway which may lead to the opening of a plasma membrane calcium channel. This is possible since a slow calcium influx was observed when fura-2 loaded cells were exposed to $sPLA_2$-I.

Unlike histamine which is stored preformed in the mast cell secretory granules, PGD_2 is rapidly synthesised and released from mast cells following activation. In this study, PGD_2 production was only observed with an $sPLA_2$-I concentration of 1 U ml^{-1} (20 min incubation). This PGD_2 could be produced by the free AA generated through $sPLA_2$-I

hydrolysis of the mast cell plasma membrane phospholipid (PC). This free AA could then be rapidly metabolised by the enzymes involved in AA metabolism inside the cell.

Alternatively, PGD_2 could be generated by activation of the intracellular $cPLA_2$ if $sPLA_2$-1 activated an intracellular signal transduction pathway through receptor binding. However, Xing *et al.* (1995) observed that short term exposure of NIH 3T3 cells to the porcine pancreatic $sPLA_2$, did not generate a significant amount of AA, whereas a 6 hour exposure did. Kishino *et al.* (1995) also reported that the porcine pancreatic $sPLA_2$ augmented PGE_2 production in rat mesangial cells over a period of 24 hours. This effect was receptor mediated and did not require the enzymatic action of $sPLA_2$-I. This suggested that the PGD_2 production observed here may not be receptor mediated.

In the present study, histamine release and PGD_2 production were observed with high concentrations of $sPLA_2$-I and within a short time span of 20 minutes. In addition, the histamine releasing activity of $sPLA_2$-I was significantly reduced with the $sPLA_2$ inhibitors employed in this study (*p*-BPB and 12-epi-scalaradial). If $sPLA_2$-I was acting through a specific receptor site on the plasma membrane, in accordance with the reports by Xing *et al.* (1995) one would expect to see histamine release in the presence of the $sPLA_2$ inhibitors. However, this was not the case and led to the suggestion that the enzymatic activity of $sPLA_2$-I on the plasma membrane may be responsible for the effects observed here. Of course, the identification of $sPLA_2$-I receptor sites on the mast cell plasma membrane would help resolve whether or not the enzymatic action of $sPLA_2$-I is solely responsible for the present effects observed here.

In contrast with $sPLA_2$-I, the maximum % histamine release observed with $sPLA_2$-II was generally not greater than 10% with resting cells. This release was reduced with the $sPLA_2$ inhibitors *p*-BPB and 12-epi-scalaradial, suggesting that the enzymatic site of $sPLA_2$-II was involved in mediating the effects observed here. The type II $sPLA_2$ enzymes display a specificity for the phospholipid PE and PS. In resting platelets, these phospholipid are found in the inner layer of the plasma membrane (Bevers *et al.*, 1983). If this membrane phospholipid composition was applied to mast cells, it may explain the low level of histamine observed in these studies as the specific substrate for $sPLA_2$-II was unavailable.

In contrast with $sPLA_2$-I, PGD_2 was produced in a dose dependent manner. This PGD_2 could be produced by hydrolysis of the membrane phospholipid (PC or PE) to yield free AA. This free AA could then be rapidly metabolised by the enzymes involved in AA metabolism inside the cell. $sPLA_2$-II could bind to a putative binding site on the mast cell plasma membrane and PGD_2 could be produced by the activation of the intracellular $cPLA_2$, if $sPLA_2$-II acted as a signalling molecule. One of the characteristics of these $sPLA_2$ receptors is their ability to internalise upon receptor binding. This internalisation could direct the action of $sPLA_2$-II into the target cell, where the substrates PE and PS are readily available. However, based on the reports by Xing *et al.* (1995) where the receptor mediated effect of the pancreatic PLA_2 enzyme was observed over a long time span of 6 hours, and Kishino *et al.* (1995) who also reported that the porcine pancreatic $sPLA_2$ augmented PGE_2 production in rat mesangial cells over a period of 24 hours, the events observed here are unlikely to be receptor mediated. Of course, the identification of $sPLA_2$-II binding sites on the mast

cell plasma membrane would help settle this.

The specificity of these $sPLA_2$ enzymes to enhance immunologically induced histamine release, suggested that they may participate in some step specifically required for IgE induced release. This enhancement was unaffected by the $cPLA_2$ inhibitor MAFP, but was reduced in the presence of the PLA_2 inhibitors *p*-BPB and 12-epi-scalaradial at concentrations that reduced the histamine releasing activity of these enzymes. This suggested that the enzymatic activity of the $sPLA_2$ was responsible for the effects observed here.

It was also observed here that both PLA_2 inhibitors, *p*-BPB and 12-epi-scalaradial inhibited the anti-IgE induced histamine release. A 14kDa type II $sPLA_2$ has been located in the secretory granules of rat peritoneal mast cells (Chock *et al.*, 1994), and is released when cells are stimulated with antigen (Murakami *et al.*, 1992a). This secreted type II 14kDa $sPLA_2$ could hydrolyse PS to yield lysoPS or another fusogenic compound and in this way augment histamine release.

Bevers *et al.* (1983) reported that in activated platelets, PE and PS were translocated to the outer layer of the plasma membrane. Cross linking of the IgE receptor molecules on the mast cell plasma membrane may lead to a membrane rearrangement event. This could make the specific phospholipid substrates PE and PS available to $sPLA_2$-II, for lipid hydrolysis. Hydrolysis of PS by $sPLA_2$-II would generate lysoPS. Both PS (10-50 $\mu g\ ml^{-1}$) and lysoPS (less than 10 μM), have been reported to enhance con A induced histamine release from rat peritoneal mast cells with lysoPS being 50

to 1,000 times more potent than PS (Goth *et al.*, 1971; Baxter & Adamik, 1977; Martin & Lagunoff, 1979; Smith *et al.*, 1979; Boarato *et al.*, 1984; Horigome *et al.*, 1986). PS can enhance antigen induced ^{45}Ca uptake in rat peritoneal mast cells (Foreman *et al.*, 1977; Ishizaka *et al.*, 1979) and lysoPS could induce ^{45}Ca influx in mouse mast cells (Lloret & Moreno, 1995). These reports would support our observations with the increased Ca^{2+} influx observed here with $sPLA_2$-II following cell activation with anti-IgE. Thus the specificity of $sPLA_2$-II to enhance immunologically induced histamine release here, suggested that it may in some way facilitate in the opening, or maintain the opening of a plasma membrane calcium channel. The mechanism(s) of action needs to be determined.

PGD_2 production was also enhanced in anti-IgE stimulated cells preincubated with the type II $sPLA_2$ enzyme purified from *Crotalus altrox* venom. Foneth *et al.* (1994) reported that the mouse mast cell line (BBMC) secrete a 14kDa type II $sPLA_2$ following antigen challenge. This $sPLA_2$-II enzyme leads to the rapid mobilisation of extracellular AA which is subsequently used for eicosanoid generation. The enhanced PGD_2 production observed here is probably due to the release of more AA from the membrane phospholipid following the enzymatic action of the secreted PLA_2-II.

A significant enhancement of immunologically induced histamine release was observed with non-releasing concentrations of $sPLA_2$-I. Release of the 14kDa type II $sPLA_2$, located in the mast cell secretory granules following cell activation, may explain the enhanced histamine release observed with $sPLA_2$-I in this study. Significant enhancement was only observed with the longer activation time with anti-IgE (10 min),

suggesting that time may be needed for secretion of the 14kDa type II $sPLA_2$ from the mast cell granules in order to mediate its effects i.e. opening of calcium channel. However, there was no enhancement observed with the Ca^{2+} signal again suggesting that time may be required for secretion of the 14kDa type II $sPLA_2$ and action on increasing the influx of calcium. There was also no significant enhancement of PGD_2 production observed with $sPLA_2$-I following activation with anti-rat IgE. The lack of enhancement observed here again maybe due to this time factor. Alternatively binding of $sPLA_2$-I to a binding site may facilitate the release or synthesis of $sPLA_2$-II.

3.10 Conclusions

To conclude, the results of these studies have indicated that the type I $sPLA_2$ purified from *Naja naja* venom, caused histamine release and PGD_2 production at high concentrations (0.5 to 10 U ml^{-1}). The mode of action proposed for this effect involved the enzymatic site of $sPLA_2$-I. The type II $sPLA_2$ purified from *Crotalus altrox* venom caused low levels of histamine release. However, PGD_2 was produced in a dose dependent manner (0.001 to 1 U ml^{-1}). The mode of action of $sPLA_2$ could involve the digestive nature of the enzyme to generate free AA or be receptor mediated and activate $cPLA_2$.

In addition to the effects observed on unstimulated mast cells, these $sPLA_2$ specifically enhanced immunologically induced histamine release. This suggested their participation in some step specifically required for anti-IgE induced release. Therefore these $sPLA_2$ enzymes may have a pro-inflammatory role in inflammation. The influx of calcium from the extracellular environment through plasma membrane calcium channels is required for immunologically induced histamine release. An increase in the $[Ca^{2+}]_i$, during the second phase of the biphasic Ca^{2+} response, following cell activation with anti-IgE was observed with $sPLA_2$-II. This was not observed with the type I $sPLA_2$. It was proposed that $sPLA_2$-II may facilitate the opening, or maintain the opening of the plasma membrane calcium channels. This could be mediated by the enzymatic nature of $sPLA_2$-II, as enhancement was reduced following *p*-BPB and 12-epi-scalaradial treatment. $sPLA_2$-II may participate in the opening of the plasma membrane calcium channels through the generation of a lipid metabolite such as

lysoPS.

Histamine release induced by anti-IgE was inhibited by the $sPLA_2$ inhibitors *p*-BPB and 12-epi-scalaradial. A role for $sPLA_2$ was proposed to be involved in anti-rat IgE induced histamine release from rat peritoneal mast cells.

3.11 Future studies

The mechanism(s) of action of the $sPLA_2$ enzymes on the mast cell plasma membrane requires further studies. The development of more specific $sPLA_2$ inhibitors both for the enzymatic site and receptor binding site would help establish what nature of these enzymes is involved in the effects observed in this study. Membrane studies would help establish the phospholipid composition of the mast cell plasma membrane. Studies to identify the lipid metabolites following phospholipid hydrolysis are also required. The present study did not provide direct evidence for the existence of selective binding sites for the $sPLA_2$ enzymes on the mast cell plasma membrane. Therefore further studies such as radioligand binding studies would help in the identification of binding sites for the $sPLA_2$ enzymes investigated here. The analysis of the biochemical signal transduction pathway(s) activated in response to $sPLA_2$ binding is another area that requires further investigation.

CHAPTER 4

EFFECTS OF SPLA$_2$ ON HUMAN AND GUINEA PIG LUNG MAST CELLS

4.1 Introduction

Most of the knowledge on the physiology and pharmacology of mast cells was initially derived from studies on the rat peritoneal mast cell. These cells can easily be obtained by direct lavage of the peritoneal cavity and can be readily purified. With the development of methods for the enzymatic dissociation of mast cells from tissues of experimental animals and man it was shown that these histaminocytes are different. Indeed these cells are known to exhibit heterogeneity in their morphological, histochemical and functional properties (for reviews see Barrett & Metcalfe, 1987; Barrett & Pearce, 1993; Irani & Schwartz, 1994). Today the idea of mast cell heterogeneity is well established and it is inappropriate to rely solely on the rat as an animal model for investigations.

The original description of mast cell heterogeneity was based on differential staining properties, between two distinct populations of mast cells identified in the rat intestine (for review see Enerbäck, 1981). Mast cells located in the lower layer of the intestinal wall resemble those found in other connective tissues and the serosal cavity. The mast cells located in the mucosa exhibit different properties. These mucosal mast cells require different fixation and staining conditions when compared with cells from connective tissues. These differential staining properties are due to the proteoglycan content found in their secretory granules within the cytoplasm. Rat peritoneal mast cells contain heparin (Yurt *et al.*, 1977) and are stained with both alcian blue and safaranin. Intestinal mucosa mast cells contain chondroitin sulphate di-B but do not contain heparin (Stevens *et al.*, 1986) and stain only with alcian blue. Based on this

finding mast cells were divided into two classes, the connective tissue mast cell (CTMC) and the mucosal mast cell (MMC). This classical classification was not so clear in other species such as in human (Strobel *et al.*, 1981; Befus *et al.*, 1985), so other criteria were required.

Mast cells can also be distinguished by their granular enzyme content. Two antigenically different serine proteases, (rat mast cell protease (RMCP) type I and II), with chymotryptic activity are differentially expressed in rat mast cells (Woodbury *et al.*, 1987). Rat peritoneal mast cells, together with cells from the skin and lung contain RMCP type I, while mast cells of the intestinal mucosa contain RMCP type II (Gibson & Miller, 1986).

Human mast cells are also distinguished by their granular protease content (for review see Irani & Schwartz, 1994). Two types of proteases have been isolated, a tryptase (tryptic specificity) and a chymase (Schwartz *et al.*, 1987a). Mast cells containing tryptase plus chymase (MC_{TC}) are predominately located in the skin and the intestinal mucosa with a minority population present in the lung (10%). Most of the mast cells in the lung (90%) and cells in the intestinal mucosa only contain tryptase (MC_T) (Irani *et al.*, 1986; Schwartz *et al.*, 1987b).

In addition to protease content, human mast cells are also heterogenous with respect to their cytokine content. Il-4 is distributed among both mast cell phenotypes, with a higher distribution in the MC_{TC} subset (85%) compared with 15% in the MC_T subset. Il-5 and IL-6 are found exclusively in the MC_T subset (Bradding *et al.*, 1995).

Morphological studies of human mast cells revealed that the secretory granules have patterns such as scrolls, crystals, particles or a mixture of all three. Lipid bodies are also present in human mast cells. Guinea pig mast cells contain basket shaped granules and may be homogeneous, granular, strandlike or crystalline in form (Dvorak *et al.*, 1983; Galli *et al.*, 1984). Rat CTMC have homogeneous granules that are uniformly electron dense (Behrendt *et al.*, 1978).

Mast cells also differ in their responsiveness to various histamine liberators. The existence of functional heterogeneity was initially observed by Enerbäck in the rat with the basic secretagogue compound 48/80. This secretagogue could degranulate connective tissue mast cells *in situ* whereas the cells in the gastrointestinal mucosa were unresponsive (Enerbäck, 1966). Peritoneal mast cells of the rat and hamster are responsive to this agent (Leung & Pearce, 1984), whereas peritoneal cells from the mouse are less sensitive (Barrett & Pearce, 1983). Tissue mast cells from the guinea pig lung and mesentery are refractory to this compound. Human lung mast cells were also unresponsive to compound 48/80. This unresponsiveness was also seen with peptide 401 and dextran in guinea pig and human lung mast cells (Pearce *et al.*, 1985).

This functional heterogeneity is also seen with antiallergic drugs. Many potential antiallergic compounds tend to show a high degree of mast cell selectivity in their action. Disodium cromoglycate is a very potent inhibitor of IgE induced histamine release from rat peritoneal mast cells (Pearce *et al.*, 1989). In contrast, it is less active against mucosal mast cells from the rat, tissue mast cells of the guinea pig and human as well as human basophils. The phosphodiesterase inhibitor, theophylline is active

against rat peritoneal mast cells (Pearce *et al.*, 1982), human cells (Barrett *et al.*, 1986) but inactive with rat intestinal mucosal mast cells (Pearce *et al.*, 1982). The β_2-adrenoceptor agonists salbutamol, formoterol and salmeterol are without effect on immunologically induced histamine release from rat peritoneal mast cells. In contrast histamine release was strongly inhibited in guinea pig and human lung mast cells (Lau *et al.*, 1994).

The heterogeneity of mast cells was investigated here by comparing the effects of the $sPLA_2$ enzymes on rat peritoneal mast cells, human lung and guinea pig lung mast cells. Rat peritoneal mast cells were employed in this study as they have been extensively characterised, are abundant and are easily isolated and purified. Guinea pig lung mast cells were also used in this study since the availability of human tissue was limited.

4.2 Methodology

The procedures described in chapter two were carried out here.

4.3 Results

4.3.1 Effects of $sPLA_2$ on the spontaneous histamine release from HLMC

Human lung mast cells were incubated with the $sPLA_2$ enzymes for 10 and 20 min. With $sPLA_2$-I there was no significant release of histamine observed with concentrations less than 10 U ml^{-1}. Approximately 10% histamine release was observed at 10 U ml^{-1} (Fig 4.1). Similar results were obtained with $sPLA_2$-II where no significant release of histamine was observed even at the highest enzyme concentration (Fig 4.2).

4.3.2 Effects of $sPLA_2$ on immunologically induced histamine release from HLMC

Anti-human IgE was used as the immunological stimulus and a final concentration of 1/100 dilution of the supplied stock was used to induce approximately 30% histamine release. Human lung mast cells were preincubated with the $sPLA_2$ enzymes for 10 min. Anti-human IgE was subsequently added and histamine release allowed to proceed for a further 10 min. At concentrations between 0.01 and 10 U ml^{-1}, $sPLA_2$-I significantly inhibited the anti-human IgE induced histamine release in a dose dependent manner (Fig 4.3 and table 4.1). As illustrated in table 4.1 the experimentally observed histamine release induced by anti-human IgE from $sPLA_2$-I pretreated cells was less than the sum of the individual effects. Differences between the observed and additive effects were compared using paired Student's *t* tests. No

significant inhibition was observed at lower concentrations of $sPLA_2$-I (0.001 to 0.005 U ml^{-1}).

The type II $sPLA_2$ enzyme also produced a dose dependent inhibition of the anti-human IgE induced histamine release. However, significant inhibition was observed at concentrations between 0.025 and 10 U ml^{-1} (Fig 4.4 and table 4.2).

Dose inhibition curves were constructed to illustrate the inhibitory potency of these enzymes (Fig 4.5). $sPLA_2$-I was more effective than $sPLA_2$-II in inhibiting histamine release, with a maximum inhibition of 72.70 $\pm$ 4.12% at 10 U ml^{-1}, whereas maximum inhibition was only 54.54 $\pm$ 4.81% with the type II $sPLA_2$.

4.3.3 Effects of $sPLA_2$ on A23187 induced histamine release from HLMC

The effects of these $sPLA_2$ enzymes on histamine release induced by the calcium ionophore A23187 were also investigated. Following preincubation with either $sPLA_2$ enzyme, the A23187 (1 μM) induced histamine release was inhibited dose dependently (Fig 4.6 and 4.7). However this inhibition was only observed at $sPLA_2$ concentrations between 1 and 10 U ml^{-1} (Fig 4.8 and table 4.3 a + b). In this study the maximum percentage inhibition was lower than that observed with anti-human IgE, 59.64 $\pm$ 10.34% for $sPLA_2$-I and 39.56 $\pm$ 8.73% for $sPLA_2$-II.

4.3.4 Effects of *p*-BPB on the inhibitory activity of $sPLA_2$-I

In order to characterise the inhibitory activity of the $sPLA_2$-I enzyme, the enzyme was pretreated with the PLA_2 inhibitor *p*-BPB before incubation with cells. In a limited series of experiments (n = 3), $sPLA_2$-I was pretreated with 10, 1 and 0.1 μM of *p*-BPB. The effects of the pretreated enzyme on anti-human IgE (1/100) induced histamine release were investigated. It was observed that 10 μM of *p*-BPB alone significantly reduced the anti-human IgE induced histamine release (Table 4.4). Concentrations of 0.1 and 1 μM of *p*-BPB did not effect the anti-human IgE induced release. It was also noted that at these concentrations, *p*-BPB did not abolish the inhibitory action of $sPLA_2$-I on immunologically stimulated human lung mast cells.

4.3.5 Effects of $sPLA_2$ on partially purified HLMC

The inhibitory activities of these $sPLA_2$ enzymes on human lung mast cells could also be influenced by the presence of other functional cells in the mixed cell population. In order to investigate this, attempts were made to partially purify the human lung mast cells population. However the relative population of mast cells was only enriched to 11.13 $\pm$ 2.06% (range 6.22 to 17.48%, n = 5), from 5.09 $\pm$ 0.75% (range 3.57 to 7.52%, n = 5) of the total nucleated cells in the dispersed lung cell suspension. Although many attempts at purification were made only five provided sufficient cells for further experiments. When cells were incubated with the $sPLA_2$ enzymes for 20 min there was no significant release of histamine observed with both cell populations (Table 4.5 a + b). However the spontaneous histamine release was elevated in the

partially purified lung mast cell population.

The effects of the $sPLA_2$ enzymes on anti-human IgE induced histamine release were also investigated. The anti-human IgE (1/100) induced histamine release was itself reduced in the partially purified cells, 9.20 ± 0.59% compared to 41.23 ± 3.31% with the mixed cell suspension (n = 3). Although the anti-human IgE induced histamine release was reduced, the inhibitory effect of these $sPLA_2$ enzymes was still observed between 1 and 10 U ml^{-1} (Table 4.6 a + b).

4.3.6 Effects of $sPLA_2$ on the spontaneous histamine release from GPLMC

Guinea pig lung mast cells were incubated with the $sPLA_2$ enzymes for 20 and 30 min. With $sPLA_2$-I concentrations between 0.1 and 10 U ml^{-1}, cells released histamine in a dose dependent manner at both time incubations (Fig 4.9). Significantly more histamine was released at the 30 min incubation than at the 20 min incubation between 5-10 U ml^{-1} ($p \leq 0.05$). The maximum histamine release was 32.82 ± 4.30% (30 min) compared to 16.93 ± 2.43% (20 min) at 10 U ml^{-1}. However, lower concentrations of $sPLA_2$-I (0.001 to 0.10 U ml^{-1}) did not seem to have any significant effect on the spontaneous histamine release. In contrast the type II $sPLA_2$ enzyme had no direct effect on the cells at any concentrations tested (0.001 to 10.0 U ml^{-1}) (Fig 4.10).

4.3.7 Effects of $sPLA_2$ on immunologically induced histamine release from GPLMC

Ovalbumin was used as the immunological stimulus at a final concentration of 0.1 μg ml^{-1}. Cells were pretreated with the $sPLA_2$ enzymes for 10 min before the addition of ovalbumin. Histamine release was then allowed to proceed for a further 20 min. The spontaneous release of histamine induced by $sPLA_2$-I itself complicates these analyses. However, no enhancement of the ovalbumin induced histamine release was observed. Concentrations of $sPLA_2$-I between 0.05 and 10 U ml^{-1}, significantly inhibited the ovalbumin induced histamine release (Fig 4.11 and table 4.7). This inhibitory effect is best illustrated in the dose inhibition curve (Fig 4.13). Between 0.5 and 10 U ml^{-1}, the % inhibition ranged between 84.83 $\pm$ 3.12% and 94.63 $\pm$ 3.97%. The type II $sPLA_2$ enzyme between 1 and 10 U ml^{-1} significantly inhibited the ovalbumin induced histamine release dose dependently (Fig 4.12 and table 4.8). This inhibitory effect was not observed at concentrations less than 0.5 U ml^{-1}. The maximum inhibition was 89.79 $\pm$ 5.05% at the highest concentration tested, 10 U ml^{-1} (Fig 4.13).

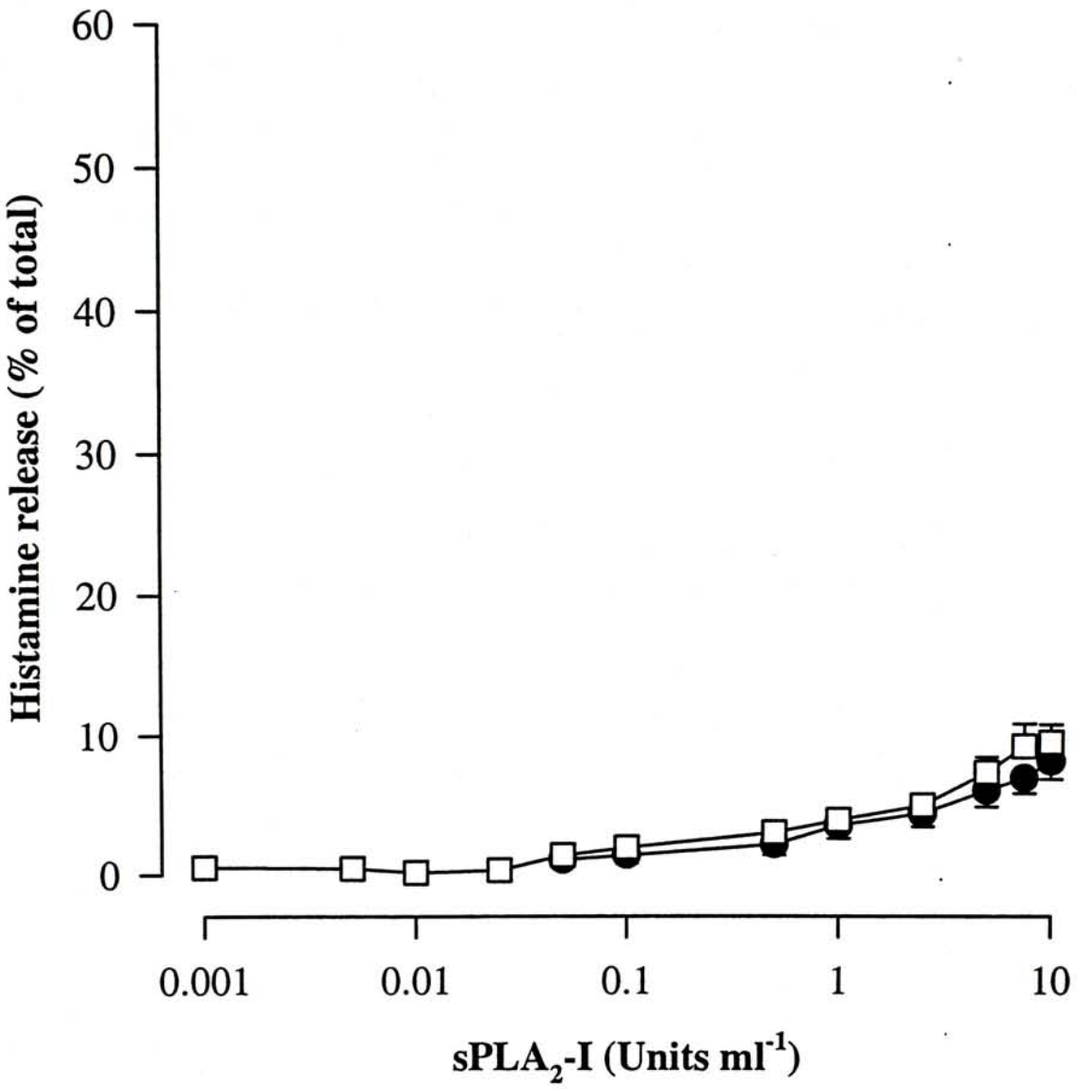

Figure 4.1 Effects of $sPLA_2$-I on the spontaneous histamine release from human lung mast cells. Cells were incubated with $sPLA_2$-I (0.05 to 10 U ml^{-1}) for 10 (●) and (0.001 to 10 U ml^{-1}) for 20 min (□). The spontaneous histamine release was 5.64 ± 0.41%. Results are given as the means ± SEM for n = 8-16.

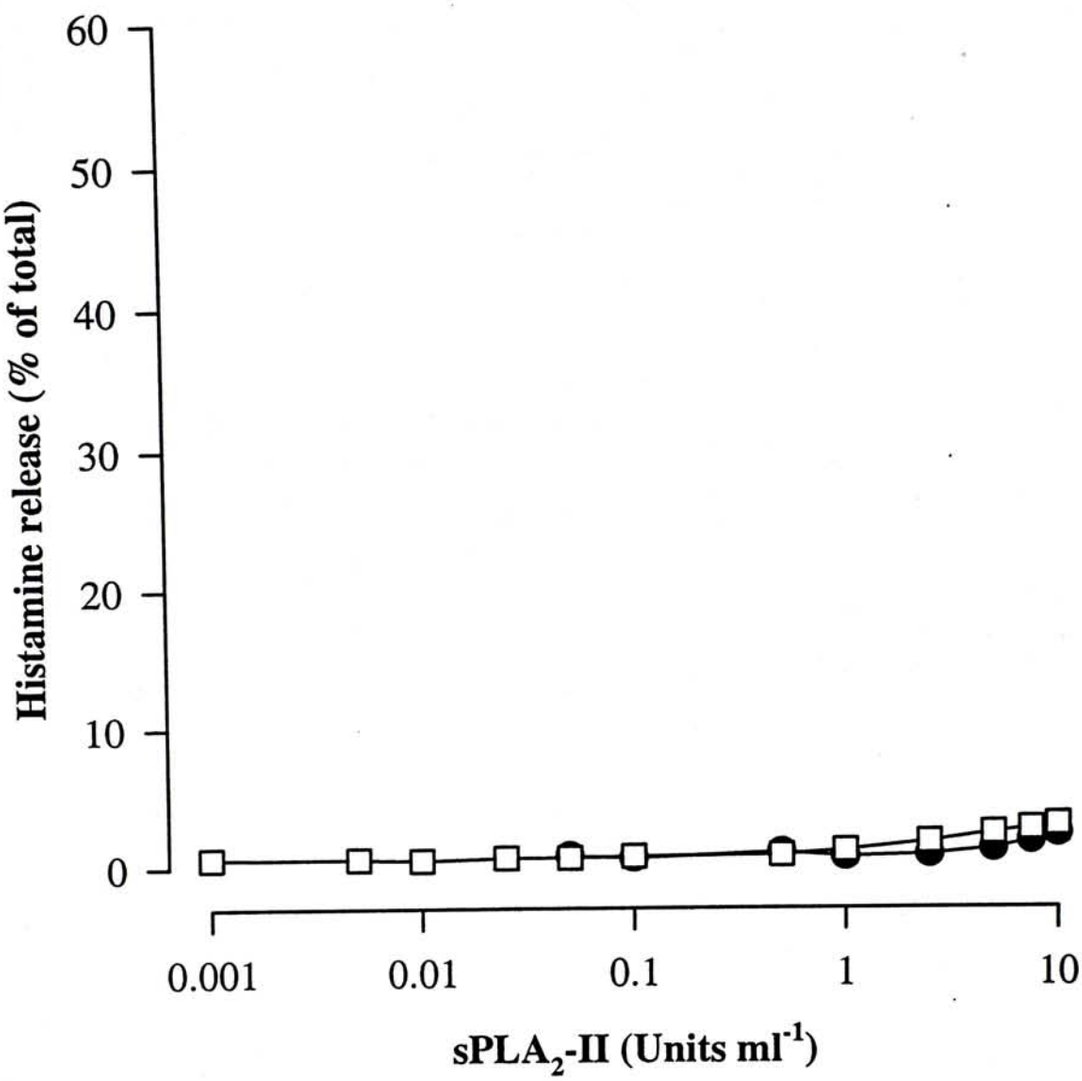

Figure 4.2 Effects of $sPLA_2$-II on the spontaneous histamine release from human lung mast cells. Cells were incubated with $sPLA_2$-II (0.05 to 10 U ml^{-1}) for 10 (●) and (0.001 to 10 U ml^{-1}) for 20 min (□). The spontaneous histamine release was 6.24 ± 0.55%. Results are given as the means ± SEM for n = 6-15.

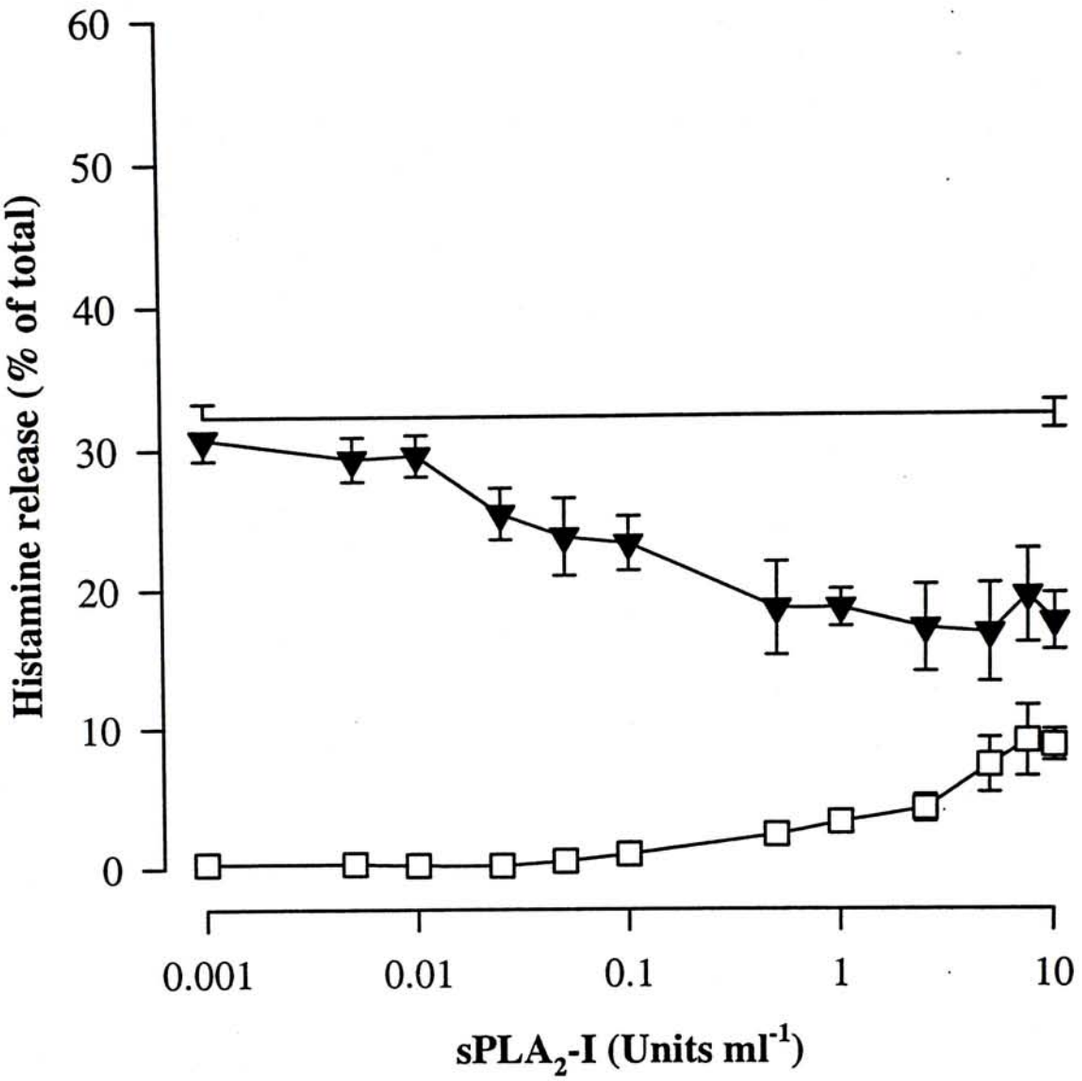

Figure 4.3 Effects of $sPLA_2$-I on anti-human IgE induced histamine secretion from human lung mast cells. Control cells were incubated with $sPLA_2$-I for 20 min (□). Anti-human IgE stimulated cells were pretreated with $sPLA_2$-I for 10 min and stimulated for a further 10 min with anti-human IgE (▼). Anti-human IgE (1/100) induced histamine release was 32.27 ± 0.99% (—). The spontaneous histamine release was 5.43 ± 0.33%. Results are given as the means ± SEM for n = 6-14.

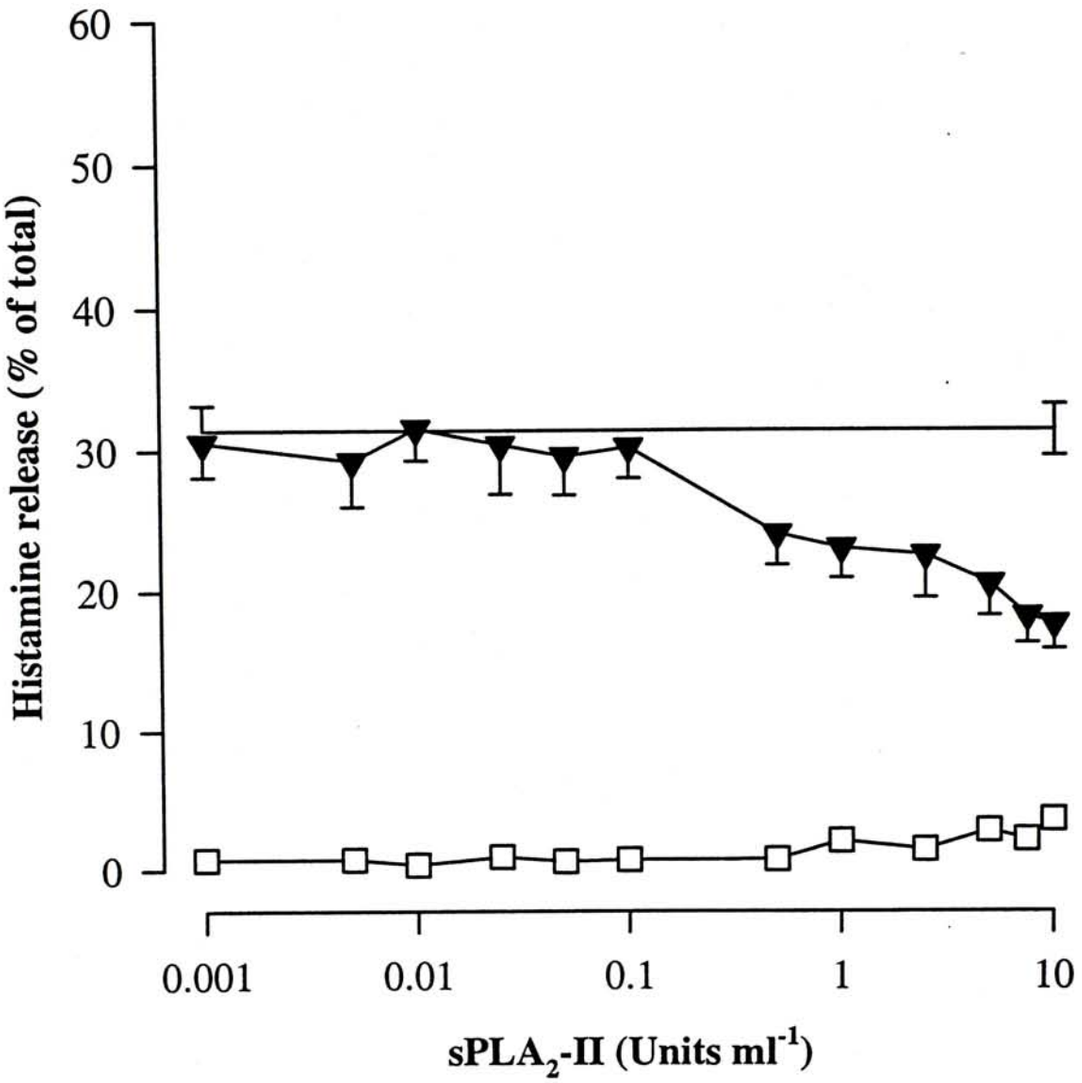

Figure 4.4 Effects of $sPLA_2$-II on anti-human IgE induced histamine secretion from human lung mast cells. Control cells were incubated with $sPLA_2$-II for 20 min (□). Anti-human IgE stimulated cells were pretreated with $sPLA_2$-II for 10 min and stimulated for a further 10 min with anti-human IgE (▼). Anti-human IgE (1/100) induced histamine release was 31.40 ± 1.81% (—). The spontaneous histamine release was 7.00 ± 0.41%. Results are given as the means ± SEM for n = 5-12.

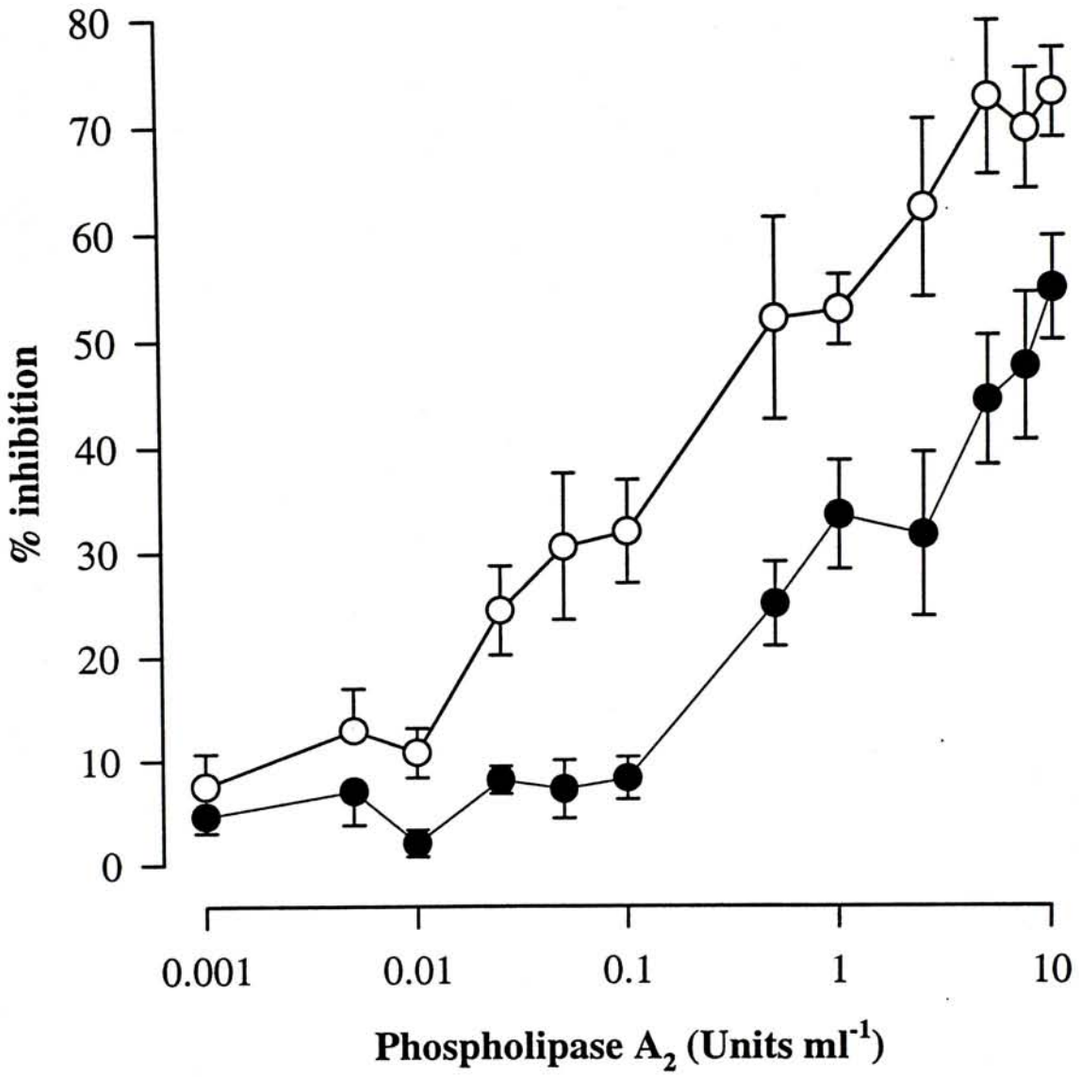

Figure 4.5 Inhibition of anti-human IgE induced histamine release from human lung mast cells by $sPLA_2$-I (○) and $sPLA_2$-II (●). Results are given as the means ± SEM for n = 5-14.

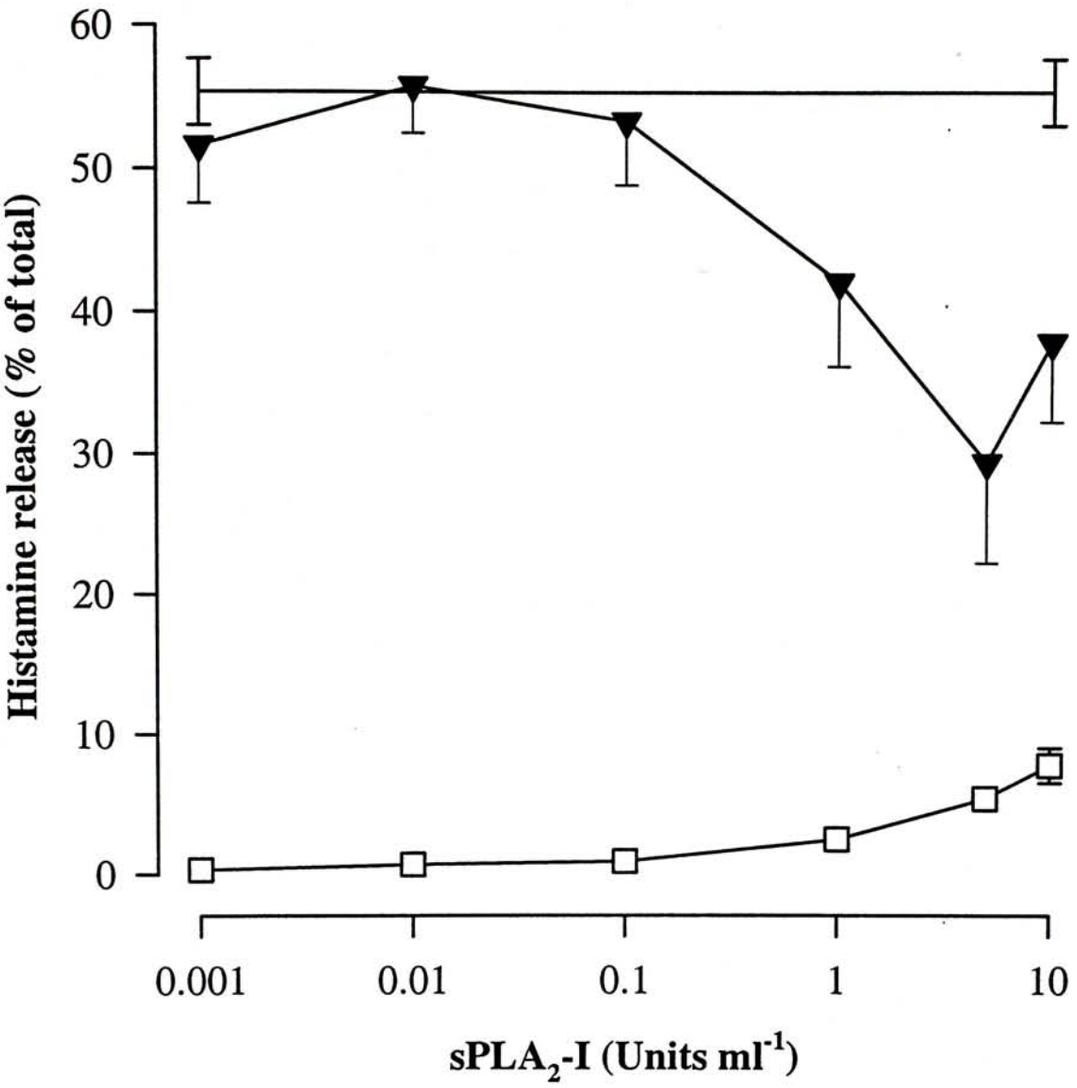

Figure 4.6 Effects of $sPLA_2$-I on A23187 induced histamine secretion from human lung mast cells. Control cells were incubated with $sPLA_2$-I (0.001 to 10 U ml^{-1}) for 20 min (□). A23187 stimulated cells were pretreated with $sPLA_2$-I for 10 min and stimulated for a further 10 min with A23187 (▼). A23187 (1 μM) induced histamine release was 55.33 ± 2.32% (—). The spontaneous histamine release was 5.61 ± 0.97%. Results are given as the means ± SEM for n = 5-8.

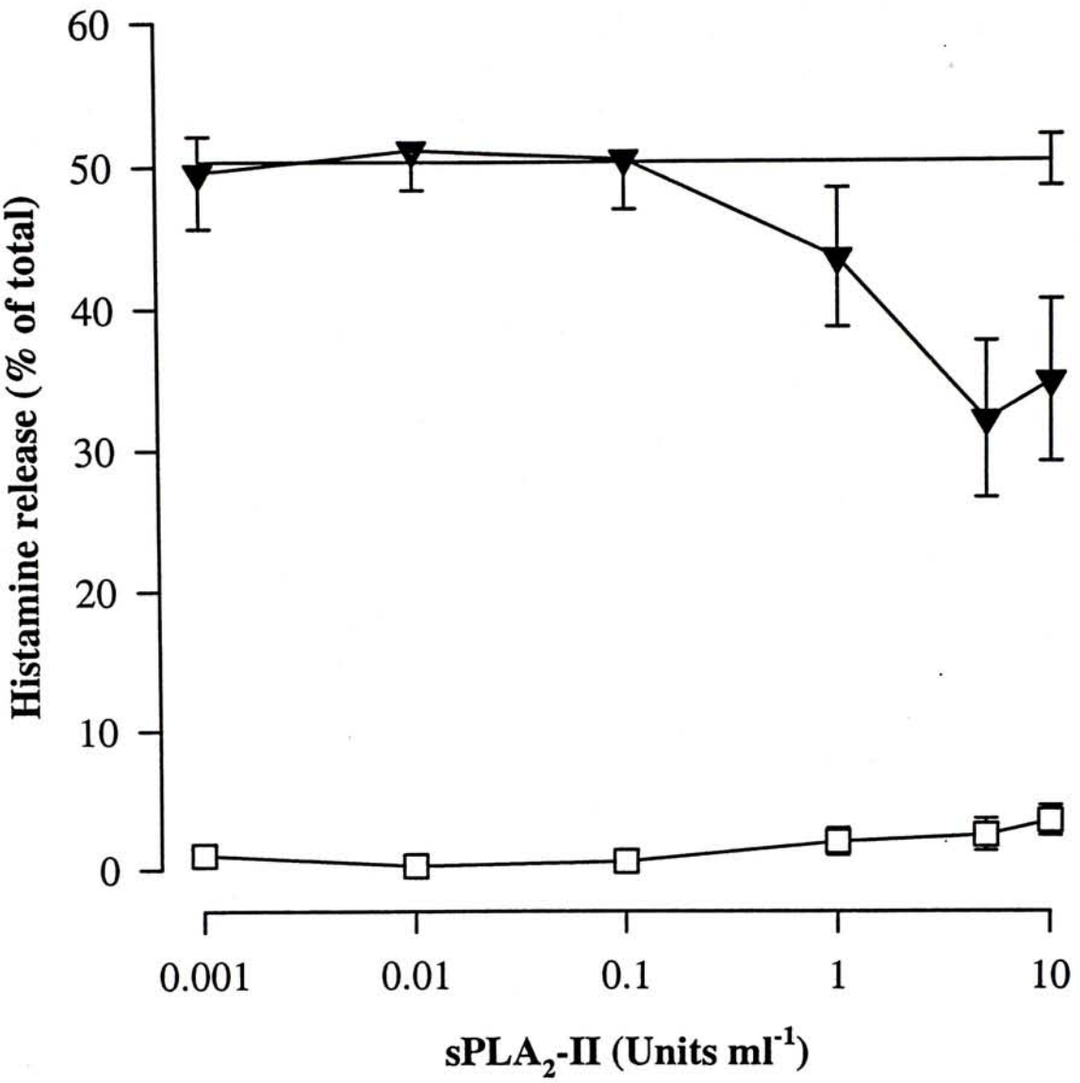

Figure 4.7 Effects of $sPLA_2$-II on A23187 induced histamine secretion from human lung mast cells. Control cells were incubated with $sPLA_2$-II (0.001 to 10 U ml^{-1}) for 20 min (□). A23187 stimulated cells were pretreated with $sPLA_2$-II for 10 min and stimulated for a further 10 min with A23187 (▼). A23187 (1 μM) induced histamine release was 50.33 ± 1.78% (—). The spontaneous histamine release was 6.62 ± 0.95%. Results are given as the means ± SEM for n = 6-9.

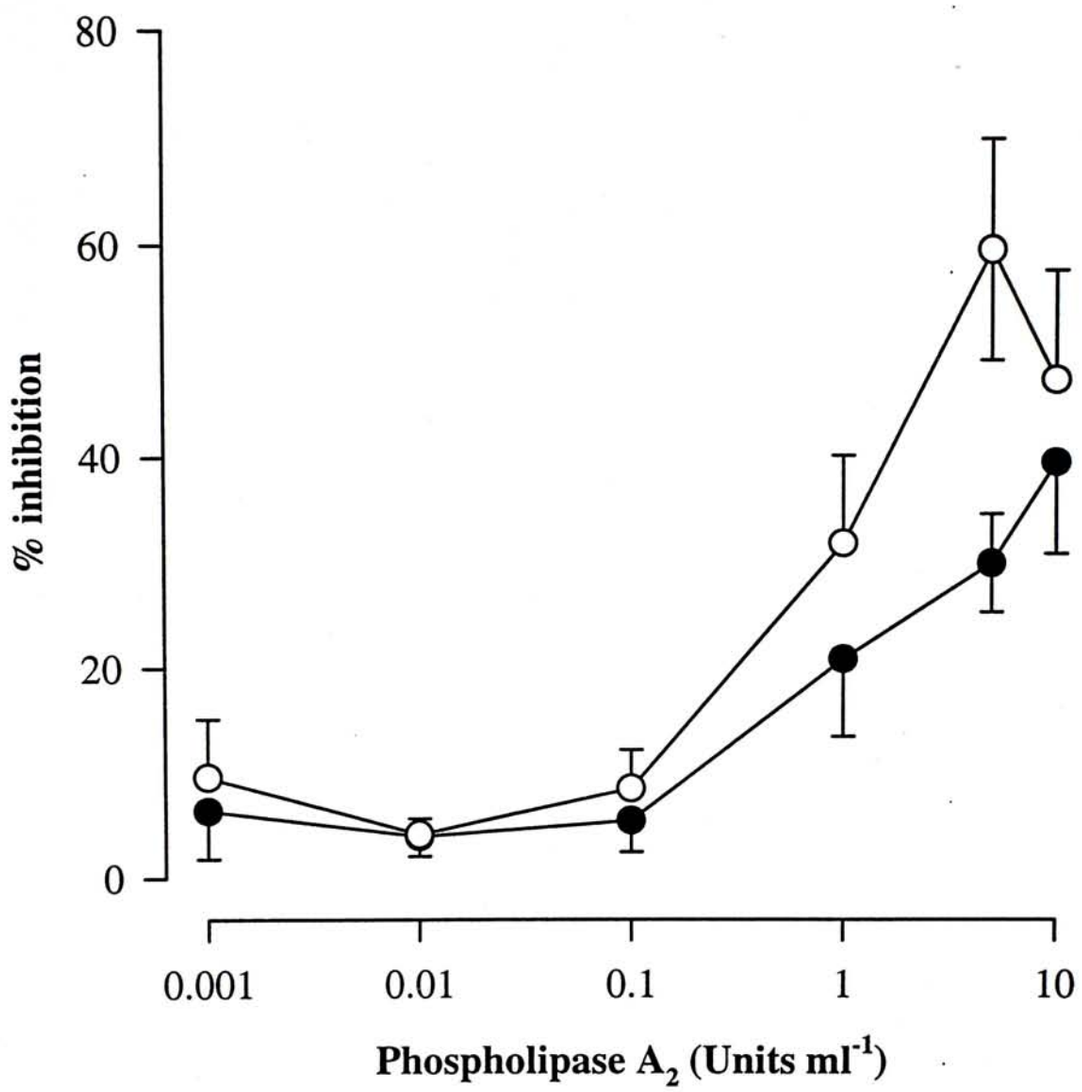

Figure 4.8 Inhibition of A23187 induced histamine release from human lung mast cells by $sPLA_2$-I (○) and $sPLA_2$-II (●). Results are given as the means ± SEM for n = 5-9.

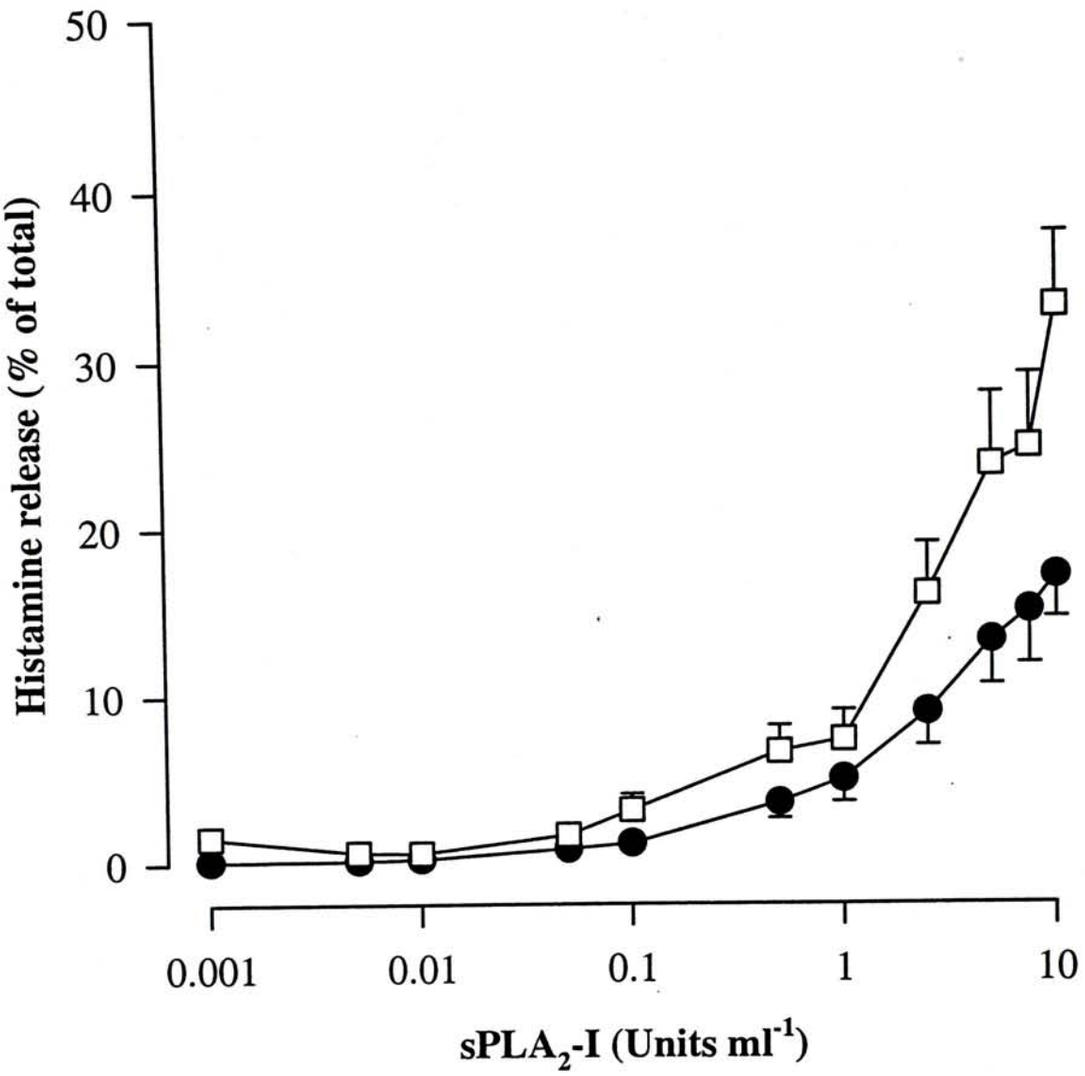

Figure 4.9 Effects of $sPLA_2$-I on the spontaneous histamine release from guinea pig lung mast cells. Cells were incubated with $sPLA_2$-I (0.001 to 10 U ml^{-1}) for 20 (●) and 30 min (□). The spontaneous histamine release was 3.30 ± 0.68%. Results are given as the means ± SEM for n = 7-12.

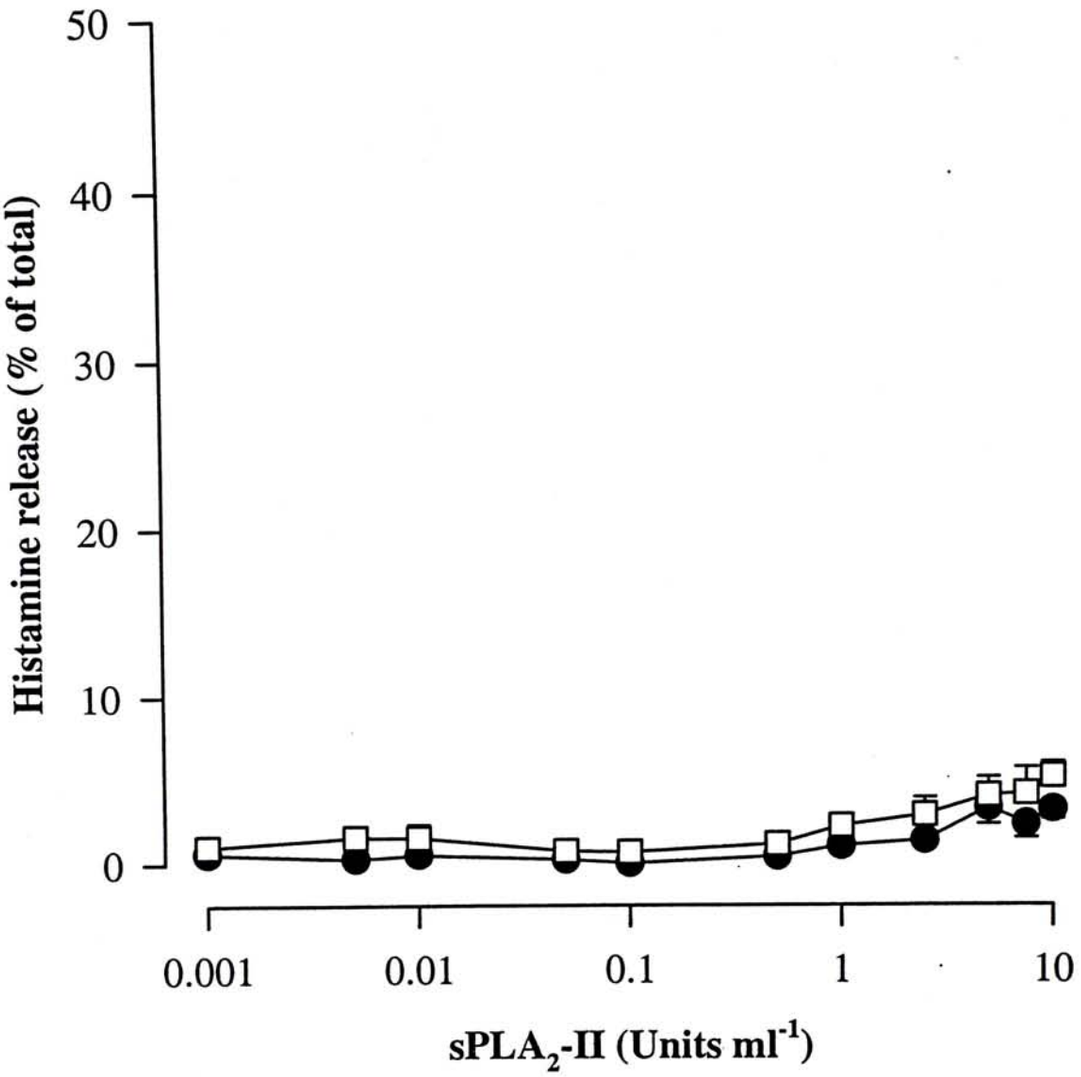

Figure 4.10 Effects of $sPLA_2$-II on the spontaneous histamine release from guinea pig lung mast cells. Cells were incubated with $sPLA_2$-II (0.001 to 10 U ml^{-1}) for 20 (●) and 30 min (□). The spontaneous histamine release was 3.52 ± 0.70%. Results are given as the means ± SEM for n = 6-12.

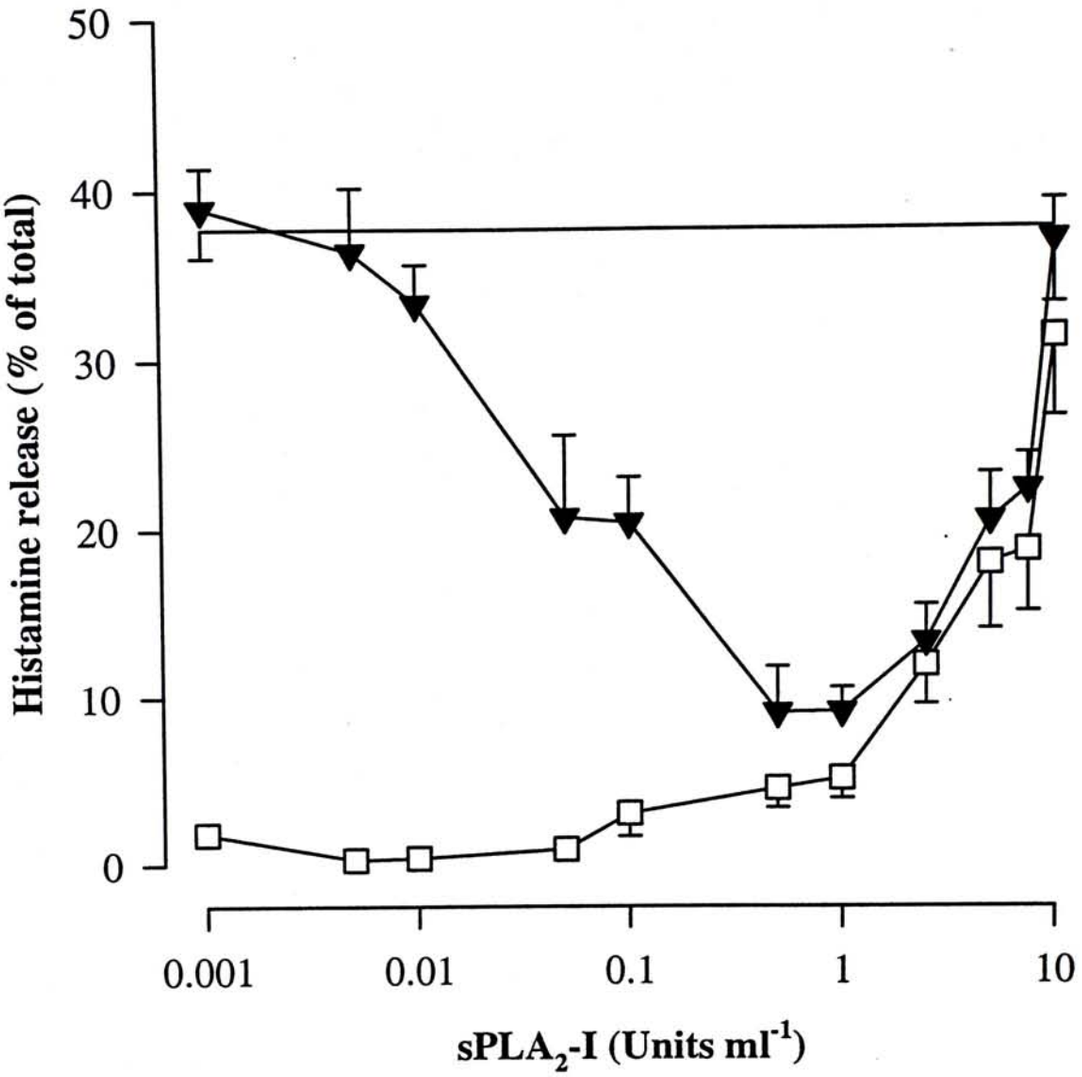

Figure 4.11 Effects of $sPLA_2$-I on ovalbumin induced histamine secretion from guinea pig lung mast cells. Control cells were incubated with $sPLA_2$-I (0.001 to 10 U ml^{-1}) for 30 min (□). Ovalbumin stimulated cells were pretreated with $sPLA_2$-I for 10 min and stimulated for a further 20 min with ovalbumin (▼). Ovalbumin (0.1 μg ml^{-1}) induced histamine release was 37.70 ± 1.67% (—). The spontaneous histamine release was 4.20 ± 0.83%. Results are given as the means ± SEM for n = 4-8.

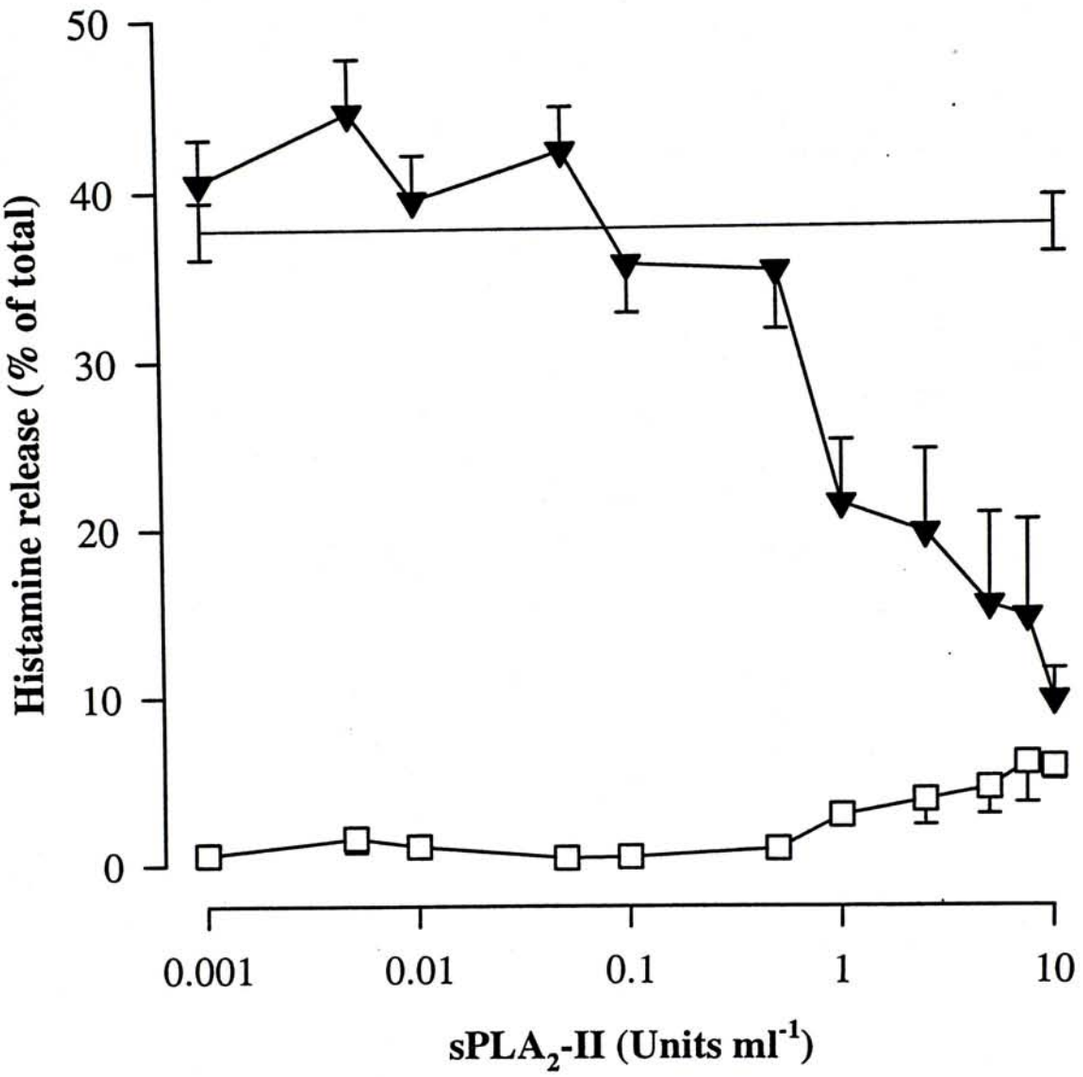

Figure 4.12 Effects of $sPLA_2$-II on ovalbumin induced histamine secretion from guinea pig lung mast cells. Control cells were incubated with $sPLA_2$-II (0.001 to 10 U ml^{-1}) for 30 min (□). Ovalbumin stimulated cells were pretreated with $sPLA_2$-II for 10 min and stimulated for a further 20 min with ovalbumin (▼). Ovalbumin (0.1 μg ml^{-1}) induced histamine release was 37.70 ± 1.67% (—). The spontaneous histamine release was 4.73 ± 0.86%. Results are given as the means ± SEM for n = 4-8.

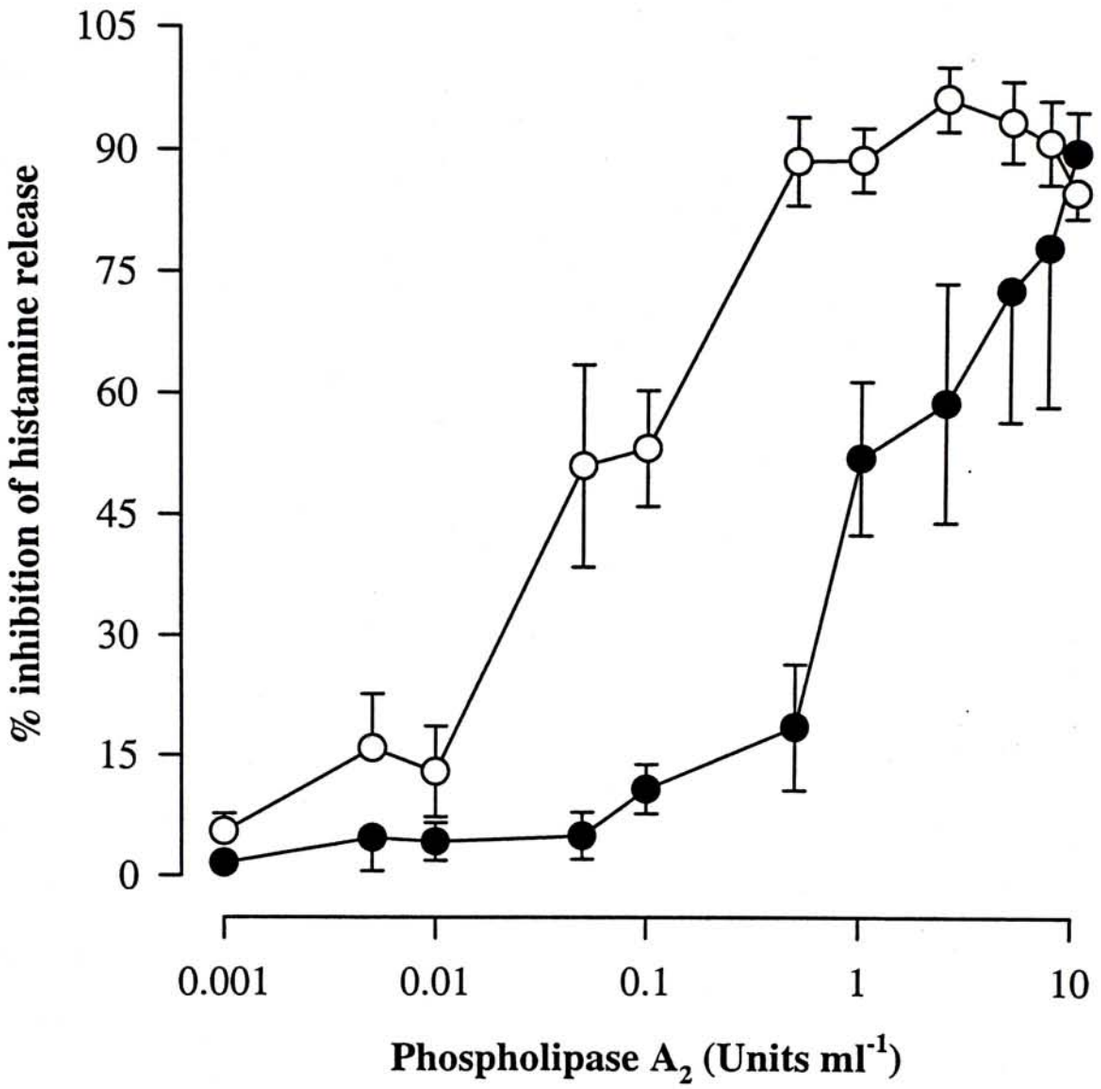

Figure 4.13 Inhibition of ovalbumin induced histamine release from guinea pig lung mast cells by $sPLA_2$-I (○) and $sPLA_2$-II (●). Results are given as the means ± SEM for n = 4-8.

Table 4.1 Comparison between histamine release induced by anti-human IgE from $sPLA_2$-I pretreated human lung mast cells with the calculated combined histamine release induced by $sPLA_2$-I and anti-human IgE. Anti-human IgE (1/100), induced histamine release was 32.27 ± 1.00%. ** = $p \leq 0.01$ as compared with the calculated combined value. Values are means ± SEM for n = 6-14.

	Histamine release (% of total)	
$sPLA_2$-I	**[$sPLA_2$-I + IgE]**	**[$sPLA_2$-I] + [IgE]**
10 Units ml^{-1}	17.59 ± 1.99**	41.00 ± 1.55
7.5 Units ml^{-1}	19.43 ± 3.32**	42.39 ± 2.50
5.0 Units ml^{-1}	16.80 ± 3.50**	45.41 ± 4.62
2.5 Units ml^{-1}	17.14 ± 3.08**	37.58 ± 1.26
1.0 Unit ml^{-1}	18.60 ± 1.33**	35.50 ± 0.92
0.5 Units ml^{-1}	18.58 ± 3.33**	35.70 ± 1.14
0.1 Units ml^{-1}	23.28 ± 1.94**	33.56 ± 1.01
0.05 Units ml^{-1}	23.77 ± 2.78**	33.81 ± 1.14
0.025 Units ml^{-1}	25.41 ± 1.83**	33.49 ± 1.11
0.01 Units ml^{-1}	29.55 ± 1.46**	32.66 ± 1.03
0.005 Units ml^{-1}	29.30 ± 1.56	33.63 ± 1.08
0.001 Units ml^{-1}	30.74 ± 1.51	32.74 ± 1.04

[$sPLA_2$-I + IgE] = histamine release induced by anti-human IgE from $sPLA_2$-I pretreated cells as observed in the experiment.

[$sPLA_2$-I] + [IgE] = combined histamine release, calculated by adding the release induced by $sPLA_2$-I alone to that induced by anti-human IgE alone for each individual experiment.

Table 4.2 Comparison between histamine release induced by anti-human IgE from $sPLA_2$-II pretreated human lung mast cells with the calculated combined histamine release induced by $sPLA_2$-II and anti-human IgE. Anti-human IgE induced histamine release was 31.40 ± 1.81%. ** = $p \leq 0.01$ and * = $p \leq 0.05$ as compared with the calculated combined value. Values are means ± SEM for n = 5-12.

Histamine release (% of total)

$sPLA_2$-II	**[$sPLA_2$-II + IgE]**	**[$sPLA_2$-II] + [IgE]**
10 Units ml^{-1}	17.61 ± 1.83**	34.99 ± 2.11
7.5 Units ml^{-1}	18.19 ± 1.94**	33.20 ± 3.06
5.0 Units ml^{-1}	20.49 ± 2.28**	35.20 ± 2.73
2.5 Units ml^{-1}	22.51 ± 3.02**	32.47 ± 2.86
1.0 Unit ml^{-1}	23.01 ± 2.13**	33.00 ± 2.24
0.5 Units ml^{-1}	24.04 ± 2.21**	31.79 ± 2.66
0.1 Units ml^{-1}	30.22 ± 2.18*	32.30 ± 2.00
0.05 Units ml^{-1}	29.57 ± 2.74**	31.62 ± 2.57
0.025 Units ml^{-1}	30.40 ± 3.49**	32.85 ± 3.50
0.01 Units ml^{-1}	31.57 ± 2.27	31.83 ± 2.20
0.005 Units ml^{-1}	29.56 ± 3.25*	31.22 ± 3.00
0.001 Units ml^{-1}	30.56 ± 2.42	31.76 ± 2.62

[$sPLA_2$-II + IgE] = histamine release induced by anti-human IgE from $sPLA_2$-II pretreated cells as observed in the experiment.

[$sPLA_2$-II] + [IgE] = combined histamine release, calculated by adding the release induced by $sPLA_2$-II alone to that induced by anti-human IgE alone for each individual experiment.

Table 4.3 (a + b) Comparison between histamine release induced by A23187 from (a) $sPLA_2$-I and (b) $sPLA_2$-II pretreated human lung mast cells with the calculated combined histamine release induced by $sPLA_2$ and A23187. A23187 (1 μM) induced histamine release was 55.33 ± 2.32% and 50.33 ± 1.78%, respectively. ** = $p \leq 0.01$ and * = $p \leq 0.05$ as compared with the calculated combined value. Values are means ± SEM for n = 5-9.

Table 4.3 (a) **Histamine release (% of total)**

$sPLA_2$-I	**[$sPLA_2$-I + A23187]**	**[$sPLA_2$-I] + [A23187]**
10 Units ml^{-1}	37.93 ± 5.60**	63.08 ± 2.19
5.0 Units ml^{-1}	29.41 ± 7.19**	61.36 ± 4.24
1.0 Unit ml^{-1}	42.11 ± 5.91**	57.81 ± 2.23
0.1 Units ml^{-1}	53.33 ± 4.46	56.64 ± 2.48
0.01 Units ml^{-1}	55.78 ± 3.28	56.03 ± 2.38
0.001 Units ml^{-1}	51.60 ± 4.02	55.68 ± 2.38

Table 4.3 (b) **Histamine release (% of total)**

$sPLA_2$-I	**[$sPLA_2$-II + A23187]**	**[$sPLA_2$-II] + [A23187]**
10 Units ml^{-1}	34.87 ± 5.75**	53.80 ± 2.44
5.0 Units ml^{-1}	32.13 ± 5.58**	50.66 ± 2.38
1.0 Unit ml^{-1}	43.59 ± 4.89*	52.33 ± 2.20
0.1 Units ml^{-1}	50.52 ± 3.51	50.90 ± 1.68
0.01 Units ml^{-1}	51.16 ± 2.79	50.56 ± 1.77
0.001 Units ml^{-1}	49.58 ± 3.90	51.32 ± 2.00

[$sPLA_2$ + A23187] = histamine release induced by A23187 from $sPLA_2$ pretreated cells as observed in the experiment.

[$sPLA_2$] + [A23187] = combined histamine release, calculated by adding the release induced by the enzyme alone to that induced by A23187 alone for each individual experiment.

Table 4.4 Effect of *p*-BPB pretreatment on the inhibitory activity of $sPLA_2$-I on histamine release induced by anti-human IgE from human lung mast cells. $sPLA_2$-I was incubated with *p*-BPB for 30 min at 37°C. The pretreated $sPLA_2$-I was then incubated with the cells for 10 min before stimulation with anti-human IgE for 10 min. Values are means ± SEM for n = 3. * = $p \leq 0.05$ as compared with the appropriate control.

Histamine release (% of total)

	***p*-BPB**			
$sPLA_2$-I	**Control**	**0.10 μM**	**1.0 μM**	**10 μM**
10 Units ml^{-1}	16.0 ± 1.5	17.6 ± 2.4	15.7 ± 2.4	5.0 ± 0.7*
1.0 Unit ml^{-1}	18.1 ± 2.6	19.2 ± 4.0	17.7 ± 2.7	5.2 ± 1.1*
0.1 Units ml^{-1}	23.1 ± 1.8	24.4 ± 1.7	22.5 ± 1.1	4.8 ± 1.4*
0.01 Units ml^{-1}	29.1 ± 0.3	29.4 ± 1.0	28.3 ± 1.2	5.9 ± 2.1*
0.001 Units ml^{-1}	32.3 ± 1.0	31.0 ± 1.6	29.5 ± 1.6	6.3 ± 2.6*
IgE (1/100)	33.0 ± 1.0	32.3 ± 0.2	29.7 ± 2.7	4.8 ± 2.1*

Table 4.5 (a + b) Comparison between the histamine release induced by (a) PLA_2-I and (b) $sPLA_2$-II, with mixed and partially purified human lung mast cells. Cells were incubated with either $sPLA_2$ enzyme for 20 min. Values are means ± SEM for n=3-4.

Table 4.5 a **Histamine release (% of total)**

$sPLA_2$-I	**Mixed**	**Partially purified**
10 Units ml^{-1}	9.01 ± 2.62	9.70 ± 2.68
1.0 Unit ml^{-1}	2.76 ± 0.12	7.14 ± 1.06
0.1 Units ml^{-1}	0.45 ± 0.45	1.81 ± 1.05
0.01 Units ml^{-1}	0.16 ± 0.16	1.21 ± 1.21
0.001 Units ml^{-1}	0.0 ± 0.0	0.86 ± 0.44
Spontaneous	4.26 ± 1.17	9.12 ± 1.44

Table 4.5 b **Histamine release (% of total)**

$sPLA_2$-II	**Mixed**	**Partially purified**
10 Units ml^{-1}	3.19 ± 1.16	1.77 ± 1.18
1.0 Unit ml^{-1}	2.79 ± 1.82	1.31 ± 0.68
0.1 Units ml^{-1}	0.67 ± 0.28	0.38 ± 0.23
0.01 Units ml^{-1}	0.49 ± 0.40	0.19 ± 0.14
0.001 Units ml^{-1}	1.41 ± 1.01	0.11 ± 0.10
Spontaneous	5.63 ± 1.77	10.54 ± 0.93

Table 4.6 (a + b) Effect of (a) $sPLA_2$-I and (b) $sPLA_2$-II on anti-human IgE induced histamine release from partially purified human lung mast cells. Control cells were incubated with either $sPLA_2$ enzyme for 20 min ($sPLA_2$). Anti-human IgE stimulated cells were pretreated with $sPLA_2$ for 10 min and stimulated for a further 10 min with anti-human IgE ($sPLA_2$ + IgE). Data are expressed for two individual experiments with the corresponding $sPLA_2$ enzymes.

Table 4.6 a **Histamine release (% of total)**

$sPLA_2$-I	**$sPLA_2$-I**	**$sPLA_2$-I + IgE**	**$sPLA_2$-I**	**$sPLA_2$-I + IgE**
10 Units ml^{-1}	15.03	18.48	6.35	11.32
1.0 Unit ml^{-1}	6.98	12.27	5.39	9.09
0.1 Units ml^{-1}	3.79	13.74	0.23	7.64
0.01 Units ml^{-1}	2.62	13.26	0.00	7.70
0.001 Units ml^{-1}	1.42	11.26	1.16	8.24
	IgE release = 10.37		IgE release = 8.69	

Table 4.6 b **Histamine release (% of total)**

$sPLA_2$-II	**$sPLA_2$-II**	**$sPLA_2$-II + IgE**	**$sPLA_2$-II**	**$sPLA_2$-II + IgE**
10 Units ml^{-1}	5.24	8.84	0.86	7.01
1.0 Units ml^{-1}	2.23	10.66	0.31	6.98
0.1 Units ml^{-1}	0.00	10.47	0.61	6.37
0.01 Units ml^{-1}	0.61	8.70	0.00	8.03
0.001 Units ml^{-1}	0.01	8.87	0.00	6.59
	IgE release = 8.53		IgE release = 8.69	

Table 4.7 Comparison between histamine release induced by ovalbumin from $sPLA_2$-I pretreated guinea pig lung mast cells with the calculated combined histamine release induced by $sPLA_2$-I and ovalbumin. Ovalbumin induced histamine release was 37.70 ± 1.67%. ** = $p \leq 0.01$ and * = $p \leq 0.05$ as compared with the calculated combined value. Values are means ± SEM for n = 4-8.

	Histamine release (% of total)	
$sPLA_2$-I	**[$sPLA_2$-I + Ova]**	**[$sPLA_2$-I]+ [Ova]**
10 Units ml^{-1}	37.07 ± 3.78**	68.77 ± 3.88
7.5 Units ml^{-1}	22.33 ± 2.09**	59.51 ± 3.80
5.0 Units ml^{-1}	20.51 ± 2.77**	58.75 ± 4.08
2.5 Units ml^{-1}	13.31 ± 2.16**	52.70 ± 2.36
1.0 Unit ml^{-1}	9.11 ± 1.43**	42.53 ± 2.67
0.5 Units ml^{-1}	9.05 ± 2.75**	45.34 ± 1.20
0.1 Units ml^{-1}	20.39 ± 2.68**	40.80 ± 1.81
0.05 Units ml^{-1}	20.71 ± 4.83*	41.72 ± 0.51
0.01 Units ml^{-1}	33.34 ± 2.28	38.09 ± 1.62
0.005 Units ml^{-1}	36.37 ± 3.78	41.08 ± 0.79
0.001 Units ml^{-1}	38.95 ± 2.39	39.53 ± 1.51

[$sPLA_2$-I +Ova] = histamine release induced by ovalbumin from $sPLA_2$-I pretreated cells as observed in the experiment.

[$sPLA_2$-I] + [Ova] = combined histamine release, calculated by adding the release induced by $sPLA_2$-I alone to that induced by ovalbumin alone for each individual experiment.

Table 4.8 Comparison between histamine release induced by ovalbumin from $sPLA_2$-II pretreated guinea pig lung mast cells with the calculated combined histamine release induced by $sPLA_2$-II and ovalbumin. Ovalbumin induced histamine release was 37.70 ± 1.67%. * = $p \leq 0.05$ as compared with the calculated combined value. Values are means ± SEM for n = 4-8.

	Histamine release (% of total)	
$sPLA_2$-II	**[$sPLA_2$-II + Ova]**	**[$sPLA_2$-II] + [Ova]**
10 Units ml^{-1}	9.77 ± 1.81*	44.29 ± 2.37
7.5 Units ml^{-1}	14.67 ± 5.69*	46.86 ± 3.06
5.0 Units ml^{-1}	15.41 ± 5.36*	45.32 ± 2.00
2.5 Units ml^{-1}	19.63 ± 4.92*	44.60 ± 1.88
1.0 Unit ml^{-1}	21.40 ± 3.74*	40.01 ± 1.81
0.5 Units ml^{-1}	35.16 ± 3.46	41.71 ± 1.06
0.1 Units ml^{-1}	35.59 ± 2.84	38.14 ± 1.67
0.05 Units ml^{-1}	42.29 ± 2.58	41.17 ± 0.70
0.01 Units ml^{-1}	39.44 ± 2.62	38.78 ± 1.96
0.005 Units ml^{-1}	44.56 ± 3.15	42.37 ± 1.27
0.001 Units ml^{-1}	40.52 ± 2.54	38.32 ± 1.82

[$sPLA_2$-II +Ova] = histamine release induced by ovalbumin from $sPLA_2$-II pretreated cells as observed in the experiment.

[$sPLA_2$-II] + [Ova] = combined histamine release, calculated by adding the release induced by $sPLA_2$-II alone to that induced by ovalbumin alone for each individual experiment.

4.4 Discussion

Mast cells isolated from the rat peritoneum, human and guinea pig lung tissues were generally unresponsive to the type II $sPLA_2$ isolated from the C*rotalus altrox* venom. The maximum % histamine release was generally not greater than 10%. This may be due to the phospholipid composition of the outer layer of the mast cell plasma membrane. As mentioned previously, Bevers *et al.* (1983) reported that in resting platelets, PC and sphingomyelin occupy the outer layer of the plasma membrane, while PE and PS are found in the inner layer. It is possible that this asymmetric lipid distribution may exist in the mast cell plasma membrane. The type II $sPLA_2$ enzymes display a substrate specificity for PE and PS (Kudo *et al.*, 1993). The location of these phospholipids in the inner layer of the plasma membrane, may explain the low level of histamine release observed here if $sPLA_2$ was mediating its effects by digesting membrane phospholipids.

The type I $sPLA_2$ isolated from the *Naja naja* venom caused the release of histamine from rat peritoneal mast cells but it had no significant effect on human lung mast cells. The maximum % histamine release from human lung mast cells was generally not greater that 10% when compared with rat peritoneal mast cells (78%). Histamine release was also observed with guinea pig lung mast cells although the maximum release was only 33%. The type I $sPLA_2$ enzymes display a substrate specificity for PC and PE. PC is located mainly in the outer layer of the plasma membrane of resting platelets (Bevers *et al.*, 1983) and this lipid distribution may apply to the mast cell plasma membrane. The histamine release observed here may be due to the enzymatic

activity of $sPLA_2$-I on the mast cell plasma membrane. The digestive nature of $sPLA_2$-I (1 and 10 U ml^{-1}) was illustrated with the human and guinea pig lung mixed cell suspensions as red blood cell lysis was observed. Human lung mast cells were unresponsive to $sPLA_2$-I and this may be due to a difference in the plasma membrane.

As already discussed in chapter 3, $sPLA_2$-I may mediate its effects by binding to specific receptor sites on the mast cell plasma membrane. $sPLA_2$-I receptor sites have been characterised in guinea pig lung parenchyma (Kanemasa *et al.*, 1992). It was reported that the porcine pancreatic $sPLA_2$-I exerted a potent contractile effect on guinea pig lung parenchyma and pleural strips (Kanemasa *et al.*, 1992; Sommers *et al.*, 1992). This $sPLA_2$-I response in guinea pig pleural strips was reduced by the cyclo-oxygenase inhibitor, indomethacin and two 5-lipoxygenase inhibitors, BW A4C and REV 6866. However, Kanemasa *et al.* (1992) observed that the contractile response in guinea pig lung parenchyma was attenuated by indomethacin and two TxA_2 receptor antagonists (+)-S-145Na and ONO3708. They proposed that some AA metabolites were involved in the response. However, mast cells were not thought to be involved, since histamine and PGD_2 levels were not significantly increased following exposure of chopped guinea pig lung tissue to this $sPLA_2$-I. Alveolar macrophages were also ruled out, since $sPLA_2$-I receptor sites were not found on these cells and TXA_2 was not released, following treatment with the porcine pancreatic $sPLA_2$-I. They proposed that some other pulmonary cells such as fibroblasts, endothelial or smooth muscle cells were mediating the observed response.

When cells were activated with the immunological stimulus anti-IgE, in the presence

of either $sPLA_2$ enzyme, a significant enhancement of the anti-IgE induced histamine release was observed with rat peritoneal mast cells. However, this enhancement was not observed with either human or guinea pig lung mast cell suspensions. The immunologically induced histamine release was dose dependently inhibited by both $sPLA_2$ enzymes, with $sPLA_2$-I being the more potent of the two. This inhibition was not restricted to immunologically activated mast cells. When $sPLA_2$-I pretreated human lung mast cells were activated with the calcium ionophore A23187, histamine release was again reduced. However, the % inhibition was much lower than that observed with anti-IgE.

As discussed in chapter 3, the specificity of the $sPLA_2$ enzymes to enhance immunologically induced histamine release in rat peritoneal mast cells, suggested that they may participate in a step specifically required for IgE induced histamine release. It was proposed that the $sPLA_2$ enzymes were involved in the opening or in maintaining the opening of a plasma membrane calcium channel since the opening of plasma membrane calcium channels is a prerequisite for immunologically induced histamine release (Sagi-Eisenberg, 1993). The inhibition observed here with the human and guinea pig lung mast cells was not specific for the immunological activation of these cells. Inhibition was also observed with ionophore induced histamine release with human lung mast cells. This would suggest that the $sPLA_2$ enzymes were not mediating their effects by blocking the influx of calcium through plasma membrane calcium channels. Instead, the $sPLA_2$ enzymes may exert their effects at some steps distal to the influx of calcium. Hydrolysis of the membrane phospholipids of activated cells may generate a lipid substance. This lipid substance may prevent fusion of the

mast cell plasma membrane with the secretory granules during exocytosis. LysoPC has been reported to inhibit mouse mast cell degranulation (Chernomordik *et al.*, 1993). The inhibitory effects observed here may also be due to the activation of a signal transduction pathway following binding to specific $sPLA_2$ receptor sites on the plasma membrane. As already discussed in chapter 3, $sPLA_2$-I receptors have been identified and cloned from a variety of tissues and cells (Lambeau *et al.*, 1994; Ishizaki *et al.*, 1994; Ancian *et al.*, 1995). It is possible that there are receptor sites on the plasma membranes of human and guinea pig lung mast cells.

Preliminary results with the PLA_2 inhibitor *p*-BPB did not remove the inhibitory action of $sPLA_2$-I on immunologically induced histamine release. However, *p*-BPB (10 μM) alone significantly reduced the anti-human IgE induced histamine release. *p*-BPB inactivates PLA_2 by alkylation of the histidine residue found in the catalytic site (Roberts *et al.*, 1977). The inability of *p*-BPB to reduce the inhibitory action of $sPLA_2$-I suggested that the catalytic site or the digestive nature of the enzyme were not involved in mediating this effect.

The different effects mediated by these $sPLA_2$ enzymes, could be due to the presence of other functional pulmonary cells. Attempts were made to partially purify the human lung mast cell population to eliminate contaminating cells. The results were unsatisfactory, as the mast cell population was only enriched to 12%. However, the observations obtained with this enriched population of mast cells were similar to the mixed cell population.

Alveolar macrophages predominate cell suspensions obtained by the enzymatic dissociation of lung tissue, but lymphocytes, neutrophils and eosinophils are also present (Schulman *et al.*, 1982; Dvorak *et al.*, 1985). These cells are capable of producing a whole spectrum of eicosanoids (Schulman *et al.*, 1981) which may be indirectly generated by mediators released from the activated mast cells. PGE_2 has been reported to have an inhibitory effect on mediator release (histamine) from dispersed human lung mast cells (Schulman *et al.*, 1982; Peters *et al.*, 1982b; Peachell *et al.*,1988). PGE_2 leads to an elevation of the intracellular cAMP level and is thought to inhibit mediator release through an antagonism of the intracellular Ca^{2+} concentration. This may explain the inhibition observed here with both the human and guinea pig lung mast cells. However, an enhancement of immunologically induced histamine release was observed with both a mixed and purified rat peritoneal mast cell population. This would suggest that the effects observed with both the human and guinea pig lung mast cells, maybe mediated through a direct action on the mast cells. The effects of these $sPLA_2$ enzymes on histamine secretion from human and guinea pig lung mast cells, has shown an inhibitory action on immunologically activated cells. However, the effect may be influenced by the presence of other pulmonary cells. In order to further investigate the biochemical events involved, there is a requirement for highly purified preparations of human and guinea pig lung mast cells.

The differences observed here may be due to the known heterogeneity among mast cells from different species (for review see Barrett & Pearce, 1993) and are not influenced by the different methods employed to obtain the mast cells (Pearce & Ennis, 1980; Barrett *et al.*, 1983; Ali & Pearce, 1985). The differences observed here

highlights the importance of studying different mast cell populations and it is inappropriate to rely solely on the rat as an animal model for investigations.

4.5 Conclusions

To conclude, these studies have indicated that the $sPLA_2$ enzymes have a pro-inflammatory role in immunologically activated rat peritoneal mast cells. This pro-inflammatory effect was specific for the rat and was not observed with human or guinea pig lung mast cells. On the contrary, an inhibitory effect was observed when mast cells from human and guinea pig lung tissue were immunologically activated in the presence of both $sPLA_2$ enzymes. This would suggest that the $sPLA_2$ enzymes would have a protective role. This role must be further investigated, in order to resolve the question of the influence of other pulmonary functional cells and establish if AA metabolites are involved. This will help establish if the anti-inflammatory role observed here is mediated through a specific action on mast cells. The functional heterogeneity observed here also emphasizes the importance of studying the precise mast cell population as the development of specific $sPLA_2$ inhibitors would abolish the protective effect observed here.

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